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von

R. H. a. Plimmer

Prof. Hermann Fischer
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EDITED BY

R. H. A. PLIMMER, D.Sc.

AND

F. G. HOPKINS, M.A., M.B., D.Sc., F.R.S.

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E. Fischer.

THE CHEMICAL CONSTITUTION OF THE PROTEINS

BY

R. H. A. PLIMMER, D.Sc.

UNIVERSITY READER AND ASSISTANT PROFESSOR OF PHYSIOLOGICAL CHEMISTRY, UNIVERSITY COLLEGE, LONDON.

IN TWO PARTS

PART II

SYNTHESIS, ETC.

SECOND EDITION

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1913

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TO

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THE MASTER OF

ORGANIC CHEMISTRY IN ITS RELATION TO BIOLOGY

697

GENERAL PREFACE.

THE subject of Physiological Chemistry, or Biochemistry, is enlarging its borders to such an extent at the present time, that no single text-book upon the subject, without being cumbrous, can adequately deal with it as a whole, so as to give both a general and a detailed account of its present position. It is, moreover, difficult, in the case of the larger text-books, to keep abreast of so rapidly growing a science by means of new editions, and such volumes are therefore issued when much of their contents has become obsolete.

For this reason, an attempt is being made to place this branch of science in a more accessible position by issuing a series of monographs upon the various chapters of the subject, each independent of and yet dependent upon the others, so that from time to time, as new material and the demand therefor necessitate, a new edition of each monograph can be issued without re-issuing the whole series. In this way, both the expenses of publication and the expense to the purchaser will be diminished, and by a moderate outlay it will be possible to obtain a full account of any particular subject as nearly current as possible.

The editors of these monographs have kept two objects in view: firstly, that each author should be himself working at the subject with which he deals; and, secondly, that a *Bibliography*, as complete as possible, should be included, in order to avoid cross references, which are apt to be wrongly cited, and in order that each monograph may yield full and independent information of the work which has been done upon the subject.

R. H. A. P.
F. G. H.

PREFACE.

THE substance Protein, which constitutes the most important part of the material basis of all animal and vegetable life, has naturally attracted the attention and energy of numerous investigators throughout the past century. Progress in the study of this subject, on account of its difficulty, has been exceedingly slow, and it is only of recent years that the discovery of new methods by Emil Fischer has enabled us to increase our knowledge to its present extent. By these methods we have been able to advance from the conception of "albumin" to its actual separation into numerous units, and also to determine their arrangement in the molecule. On this account a monograph embodying the results of the most recent investigations, together with their connections with the work of the other and earlier investigators, needs no excuse for its appearance, as the subject is now being studied in every direction.

On account of the mass of material connected with the subject, this monograph has exceeded the proposed limit in length, and consequently it has become necessary to divide it into two parts:—

I. { The Chemical Composition of the Protein Molecule.
   { The Chemical Constitution of its Units.

II. The Synthesis of the Proteins.

R. H. A. P.

PREFACE TO SECOND EDITION.

THREE years have elapsed since the appearance of the first edition of this monograph. During these years there has been continuous work on the chemistry of the proteins. What is the result? No startling discovery has been made, but we have a better and deeper view of what is known. Emil Fischer, our master, has entrusted his ester method to his former pupil, Abderhalden, and has himself studied the more difficult question of the synthesis of the proteins (the polypeptides) and the peculiar optical properties of the amino acids. These properties, he thinks, give us an insight into chemical substitution in general.

Abderhalden has analysed, it would seem, as many proteins as possible, and has also devoted much labour to the polypeptides and the action of enzymes; in fact, the amount of work which he has accomplished is extraordinary. His analyses of the proteins are of great physiological interest, and are for the purpose of ascertaining whether proteins of similar origin are identical and whether they differ at different times of life. His chemical work on the polypeptides is intimately connected with the synthesis of the proteins and must lead later to a knowledge of those ill-defined substances, the proteoses and peptones. His physiological work on these compounds is concerned with the enzyme action of various tissues and the problem of nutrition.

Osborne is giving us as complete an analysis of his vegetable proteins as he has given us of their preparation and properties. From his careful and critical study of the data obtained by the ester method he comes to the conclusion that only about 5-15 per cent. of some of these proteins remains to be accounted for.

Our methods of analysis of the proteins have been extended and improved by Van Slyke, who, in conjunction with Levene, has also introduced a method for the investigation of what is termed the "leucine" fraction.

There were only a few gaps in the section devoted to the synthesis of the amino acids; one has been completely filled by Pyman, who has synthesised histidine.

In this new edition a more detailed account of the methods of analysis of the proteins has been given, and the subject matter of Part II. has been rearranged so as to give a more complete idea of the problem of their synthesis. The omissions of the previous edition which have been kindly pointed out to me have been inserted, and it is hoped that this edition will have a larger usefulness than the first.

R. H. A. P.

September, 1912.

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THE CHEMICAL CONSTITUTION OF THE PROTEINS.

PART II.

INTRODUCTION.

Earlier Ideas.

A CONCEPTION of the composition of a complex substance must precede its synthesis. Mulder's supposition that albumin, fibrin, caseinogen and other similar substances were combinations of a hypothetical radicle, which he termed protein, with sulphur and phosphorus in various proportions, was overthrown by the work of Liebig and his pupils.

Liebig was the first to recognise that the composition of complex compounds could not be ascertained by elementary analysis but only by the analysis of their decomposition products. He was also the first to regard proteins as combinations of amino acids. At this time only three amino acids, glycine, leucine and tyrosine, were known to Liebig as decomposition products of proteins; before his death in 1873 the number of units known had risen to eight, and since that time the number has increased to seventeen.

Condensation of Amino Acids by Heating. Anhydrides.

Before a proper comprehension of the mode of union of the amino acids in proteins was possible, condensation products obtained by heating, or by distilling, amino acids had already been described:—leucinimide from leucine, lactimide from alanine and anhydrides of aspartic acid, phenylglycine, phenylalanine and sarcosine; but only in the case of the anhydride of sarcosine was the constitution determined. Condensation products were also prepared by heating together amino acids and urea. Several of these products resembled proteins in their properties: they were amorphous, easily soluble in water and gave the

biuret reaction, but they had no other characteristic properties. The anhydrides of aspartic acid were more fully investigated by Schiff in 1897-1899, and he assigned formulæ to the various products. These formulæ show that there existed at this time no concrete idea of the mode of combination of the amino acids in a protein molecule.[1]

Several remarkable compounds were described by Curtius during his investigations of the derivatives of glycine from 1881-1888. They were:—

A, two anhydrides which resulted when glycine ester was kept for some time; the one anhydride was insoluble and was proved to be derived from two molecules of glycine; the other anhydride was readily soluble and gave the biuret reaction; hence it was termed the biuret base. This base and other anhydrides of glycine formed the subject of research by several other observers. The first anhydride of Curtius has been shown to be a compound which we now term glycine anhydride and the biuret base has been shown to be the ester of triglycyl-glycine.

B, two acids of high molecular weight obtained by the action of benzoyl chloride upon the silver salt of glycine; the one was found to be hippuryl-glycine or benzoyl-glycyl-glycine, the first definite compound known which contained two amino acid radicles in combination; the other had the formula $C_{10}H_{12}N_2O_4$.

The composition of this compound could not then be ascertained but was proved many years later when Curtius returned to the subject in 1890. With the help of his pupils he subsequently prepared a series of compounds in which the amino acids are linked together. These compounds all contain a benzoyl group in their molecule, and have consequently not attracted the attention of other workers; their mode of synthesis is peculiar and they undergo many interesting reactions (pp. 19-26).

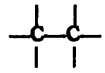
Hofmeister's Summary.

No definite theory of the mode of combination of the amino acids in a protein seems to have been enunciated before the beginning of the twentieth century, although the same idea was already in the minds of several workers at this time. Hofmeister summarised and criticised the exact position of our knowledge of the proteins in 1902 as follows:—

[1] Textbooks on organic chemistry scarcely mentioned the chemistry of the proteins, and some textbooks on physiology published at this time gave fantastic representations for the formula of a protein.

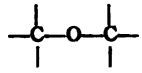
There are three ways in which we can conceive that the amino acids are combined together in the protein molecule:—

I. The carbon atoms can be linked together directly:



Under these conditions the protein molecule would be a huge branched carbon chain; its decomposition into larger and smaller complexes, especially by the action of ferments, is difficult to understand; and further, the decomposition of chains of carbon atoms even by the action of the very active enzyme trypsin, has never been observed. This mode of combination is therefore very unlikely.

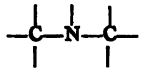
II. The carbon atoms can be linked together by an oxygen atom:



An ether-like combination of the amino acids was suggested by Nasse from the analogy between the hydrolysis of proteins by enzymes and that of the carbohydrates and fats. On account of the small number of hydroxyl groups in the molecules of the amino acids, which is limited to those contained in tyrosine, serine and oxyproline, the principal method of combination cannot be of this nature.

For the same reasons an ester-like combination of the carboxyl group of an amino acid with a hydroxyl group cannot occur, nor is the acid anhydride method of combination possible. A further reason against this method is the strongly basic character of such compounds, as was first shown by Curtius in the case of glycine ester.

III. The carbon atoms can be linked together by a nitrogen atom:



This structure admits of several different arrangements, of which the three following are the most likely:—

$$\underset{(a)}{\text{—CH}_2\text{—NH—CH}_2\text{—}} \qquad \underset{(b)}{\text{—CH}_2\text{—NH—CO—}} \qquad \underset{(c)}{\text{—CH}_2\text{—NH—C(NH)—}}$$

A linking as in Scheme (*a*) is found, for example, in proline; but it cannot occur to any large extent, since if the union between amino acids be thus effected the molecule would become strongly acid in character, owing to the free carboxyl groups.

A linking as in Scheme (*c*), which is that of guanidine, occurs in arginine, and this is the only compound amongst the decomposition products of proteins in which this arrangement of the atoms occurs:

such linkings therefore cannot play a great part in the constitution of the molecule.

A large number of significant facts support Scheme (*b*) as the most feasible method for the combination together of the amino acids.

(*a*) The products of hydrolysis.

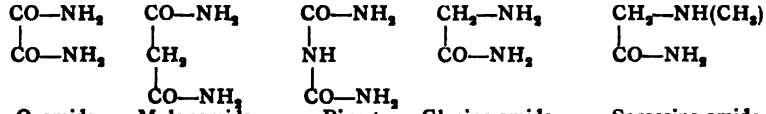
(i) A small proportion of the total nitrogen of the protein molecule is liberated on hydrolysis as ammonia; this points to the presence of this linking in the form of acid amide, —CO—NH_2, combinations.

(ii) The greater portion of the total nitrogen—about 90 per cent.—is present in the products of hydrolysis in the form of amino (NH_2) groups. These groups are not present in the protein molecule as such, since by the action of nitrous acid on the protein the amount of nitrogen liberated is very small in amount, and in no way corresponds to the amount obtainable if the greater part of the nitrogen be present in the form of amino groups.

It must therefore be assumed that the NH_2 groups of the end products exist in the protein molecule in the form of NH groups.

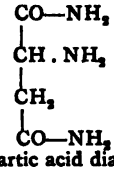
(*b*) The biuret reaction.

The biuret reaction, which is one of the chief characteristics of a protein, is according to Schiff given by those substances which contain two CO—NH complexes, or two CS—NH or C(NH)—NH complexes, and under certain conditions two —CH_2—NH complexes, combined together directly, or by a carbon atom, or by a nitrogen atom, *e.g.*,



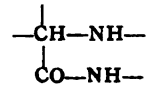
Oxamide | Malonamide | Biuret | Glycine amide | Sarcosine amide

and also

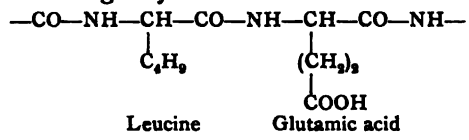


Aspartic acid diamide

All these compounds give very intense biuret reactions. The presence of



groups in the protein molecule is therefore very probable. Such groups occur when amino acids, *e.g.*, leucine and glutamic acid, are combined together in the following way :—



and they are repeated when another amino acid is again combined in this manner.

(*c*) The combination of amino acids by the formation of

$$CH_2—NH—CO$$

groups is supported by the facts observed in the living body.

Hippuric acid $C_6H_5 . CO—NH . CH_2 . COOH$ is formed from benzoic acid and glycine by the kidney, and the bile acids are presumably substances of similar constitution.

(*d*) The condensation products of amino acids, obtained by Schaal, Grimaux and by Curtius, many of which give the biuret reaction, support the above contention.

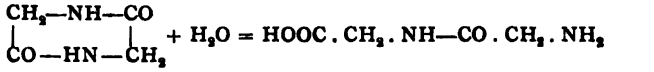
The Polypeptides.

Our knowledge of the structure of the protein molecule is due to the systematic researches of Emil Fischer and his pupils which were begun in 1901 and are being continued at the present time. To them also is due the proof of the accuracy of Hofmeister's view that the acid amide form of combination of the amino acids is the principal one in the protein molecule.

Fischer has termed the combinations of the amino acids which he has prepared, *the polypeptides*. This designation is in imitation of the nomenclature adopted for carbohydrates where we differentiate between mono-, di-, tri-, poly-saccharides according to the number of units, and it contains the word peptone because these synthetical compounds have very similar properties to the peptones derived from proteins by hydrolysis by pepsin and weak acids and alkalies. The natural peptones in all probability consist of a mixture of polypeptides.

Fischer has devised three general methods for the synthesis of the polypeptides :—

1. By boiling the anhydride of glycine with concentrated hydrochloric acid he found that the diketo- or diacipiperazine ring was split and that the hydrochloride of a base $C_4H_8N_2O_3$ was formed :—



This compound contained two amino acid radicles joined together and was called glycyl-glycine, the group

$$NH_2 . CH_2 . CO$$

being termed the glycyl group; it was the first dipeptide known.

The anhydrides, or diketopiperazines, of alanine and of leucine were afterwards prepared, and both gave on hydrolysis the corresponding dipeptides, alanyl-alanine and leucyl-leucine.

Difficulties were, however, experienced with the preparation of the anhydrides of other amino acids, but a few years later he found that they could be readily obtained by heating the esters of the amino acids to 150-180° C. and that the diketopiperazine ring was very rapidly split by the action of dilute alkali at the ordinary temperature. The anhydrides and dipeptides of alanine, leucine, phenylalanine and tyrosine were thus obtained. The dipeptides of the diamino acids, lysyl-lysine and histidyl-histidine, and of serine have up to the present time only been made by this method. The method does not lend itself to the preparation of anhydrides or dipeptides containing two different amino acids. These compounds can, however, be prepared indirectly (p. 29).

The properties of the amino acids recur in the dipeptides: they are easily converted into their esters, and higher polypeptides are formed when these esters are heated.

2. By introducing an α-halogen acyl radicle into the molecule of an amino acid and then treating this compound with ammonia, the halogen atom is replaced by the amino group and a dipeptide is formed thus :—

Chloracetyl tyrosine is obtained by the action of chloracetyl chloride upon tyrosine :—

$$Cl . CH_2 . COCl + NH_2 . \underset{\displaystyle COOH}{\underset{|}{CH}} . CH_2 . C_6H_4OH = HCl + Cl . CH_2 . CO{-}NH . \underset{\displaystyle COOH}{\underset{|}{CH}} . CH_2 . C_6H_4OH$$

From this compound glycyl-tyrosine is formed by the action of ammonia :—

$$Cl . CH_2 . CO{-}NH . \underset{\displaystyle COOH}{\underset{|}{CH}} . CH_2 . C_6H_4OH + NH_3 = HCl + H_2N . CH_2 . CO{-}NH . \underset{\displaystyle COOH}{\underset{|}{CH}} . CH_2 . C_6H_4OH$$

If this reaction be repeated, using a dipeptide in place of an amino acid, a tripeptide results; a tetrapeptide using a tripeptide and so on.

Using various halogen acyl chlorides a large number of di-, tri-, tetra-, and penta-peptides have been prepared. The method has its limitations as all the corresponding halogen acyl radicles are not

known and it only admits of the chain being lengthened in one direction, namely on the side of the amino group.

3. It was of very great importance to be able to lengthen the chain on the side of the carboxyl group. This is possible only by means of the acid chloride of the amino acid. At the commencement of Fischer's work the acid chlorides of the amino acids were unknown, but it was discovered that the acid chloride of the halogen acyl derivative of an amino acid could be prepared; these compounds react with the esters of amino acids in the following way:—

chloracetyl-glycyl chloride + alanine ester give chloracetyl-glycyl-alanine ester.

Subsequent hydrolysis of the ester and treatment with ammonia gives the tripeptide.

Higher polypeptides are formed if esters of the di-, tri-peptides are used in the place of the amino acid ester.

Fischer ultimately succeeded in preparing the acid chlorides of the amino acids so that any possible combination of the amino acids can now be made by this method.

These methods have been used for the synthesis of several higher polypeptides, such as two hexapeptides, a heptapeptide, an octapeptide, a dodecapeptide and even an octadecapeptide which contains eighteen amino acid radicles combined together. They consist principally of glycyl radicles; the dodecapeptide contains eleven glycyl radicles and one leucyl radicle; the octadecapeptide fifteen glycyl radicles and three leucyl radicles. Though such long chains composed of glycine probably do not exist in a natural protein, with perhaps the exception of gelatin which contains about 20 per cent. of glycine, their preparation has shown the possibilities of synthesis by these methods.

Glycine was used as unit in these complex substances partly because it can be obtained easily, and partly because stereoisomeric compounds cannot occur in these synthetical compounds. Glycine is readily prepared by synthesis; this is not the case with most of the other amino acids, many of which are most easily obtained by the hydrolysis of proteins. Glycine is optically inactive and is used in preference to the racemic amino acids since four stereoisomeric forms of a dipeptide containing optically active units can exist and the number is greater as the length of the chain increases.

Up to the present time over two hundred polypeptides have been synthesised, and a great number of the simpler ones consist entirely of optically active units.

The structure of the polypeptides is known from their synthesis, but a study of their properties has made it appear probable that other structures can occur. Their nomenclature in the case of optically active compounds is simple, *e.g.*, l-leucyl-d-alanyl-l-tyrosine, but where racemic forms are concerned it is more difficult; before their configuration was established Fischer had simply labelled them as the A and B forms, the A form denoting the more insoluble.

In their properties the polypeptides closely resemble the proteins: the biuret reaction is positive as soon as the compounds contain three units and the other colour reactions are positive if the compound contains the particular unit: the higher polypeptides are precipitated by tannic acid, phosphotungstic acid and other alkaloidal reagents: some can also be salted out by saturation with ammonium sulphate. The octadecapeptide has the highest molecular weight of any compound as yet obtained by synthesis, and had it been discovered in nature it would undoubtedly have been regarded as a protein.

Not only do the polypeptides closely resemble the proteins in properties but also in their behaviour to enzymes. The majority of the polypeptides are readily hydrolysed by the proteoclastic enzymes, with the exception of pepsin. Several interesting and remarkable factors have been observed in this connection; only those polypeptides containing the natural isomer of the amino acid are hydrolysed, and in the case of racemic compounds the hydrolysis is effected asymmetrically; the hydrolysis also depends upon the order of combination of the units, especially in the case of the enzyme trypsin.

The Structure of Proteins.

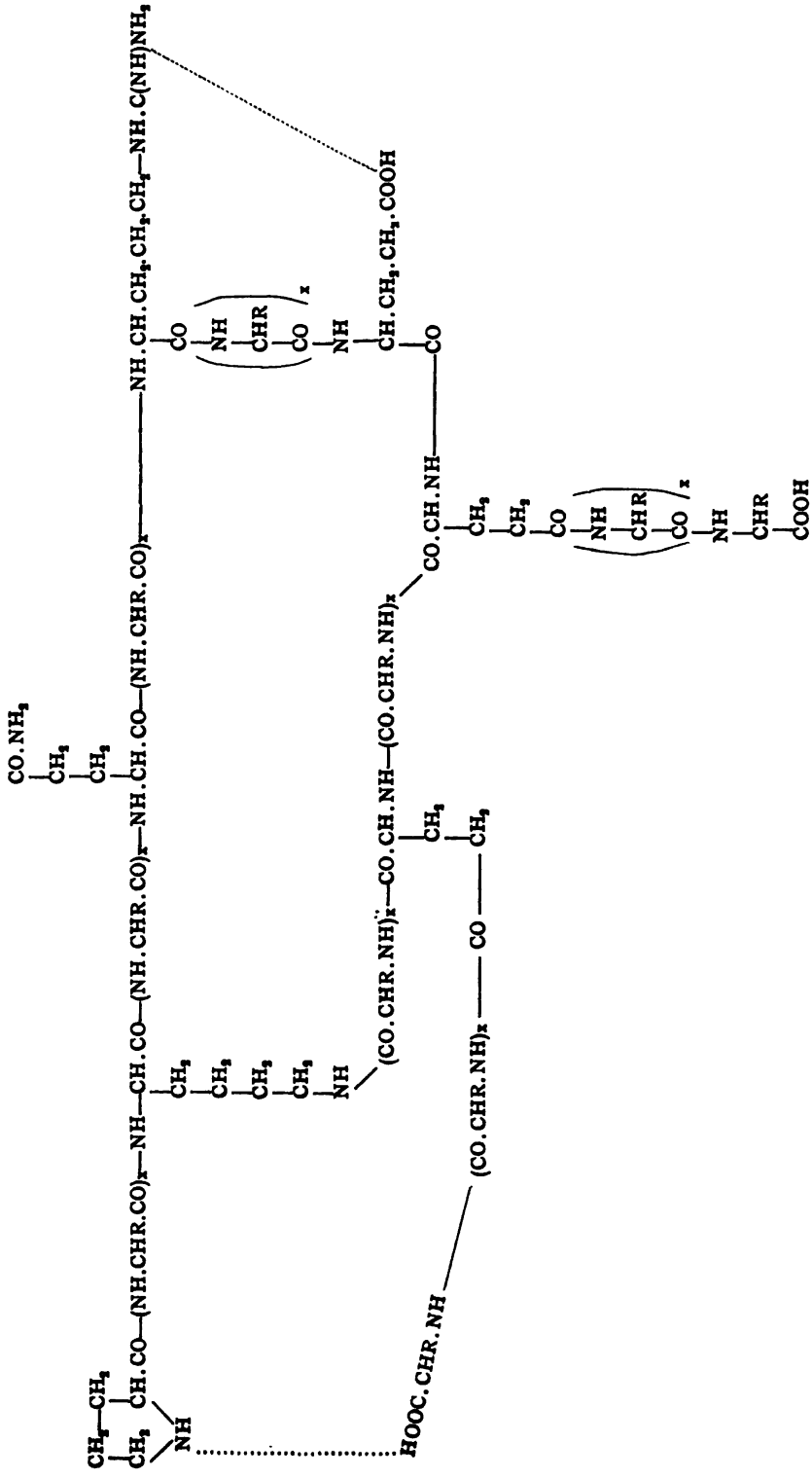
The close resemblance of the synthetical polypeptides to the natural proteins shows most conclusively that the protein molecule is built up of amino acids combined together in the form of acid amides, that they have in fact the following structure:—

$$NH_2 . CHR . CO—(NH . CHR . CO)_x—NH . CHR . COOH.$$

The arrangement of the amino acids in the synthetical polypeptides is known by their method of preparation. Isomers occur amongst the dipeptides, *e.g.*, glycyl-alanine and alanyl-glycine. The total number of possible dipeptides is 272, and this number increases as we proceed to consider tri-, tetra-, penta-peptides, etc. On account of this large number of isomers amongst even simple polypeptides it is not conceivable how a natural protein can be synthesised, except by chance, until it has been ascertained in what order the various units are combined with one another. The action of trypsin upon the dipeptides

points to one isomer being present in proteins and not the other, but these data are of little value since other enzymes may hydrolyse those combinations not attacked by trypsin. Definite information is given by the isolation and identification of polypeptides resulting from incomplete hydrolysis of protein. The separation of these compounds also affords further proof of the acid amide structure of the protein molecule. Several polypeptides have now been obtained from proteins, and we know, therefore, the arrangement of some of these units in the proteins from which they have been derived. The position of certain units in a protein molecule is shown by the action of reagents on the protein followed by complete hydrolysis; nitroarginine has been prepared from nitroclupeine; the guanidine grouping in arginine is therefore probably not used in the linking together of amino acids. An amino grouping which is not in combination with another amino acid will be attacked by nitrous acid; subsequent hydrolysis and analysis will show the absence of this amino acid. Proteins are readily racemised by treatment with alkali, but the racemisation is not complete; subsequent hydrolysis gives a mixture of optically active and optically inactive amino acids: according to Dakin the optically active acids will stand at the end of a chain (p. 87).

There is no reason to suppose that the amino acids in a protein are combined in any other way than as acid amides, for we have no evidence of the presence of ester or ether linkages between oxyamino acids nor of the imide form of combination. But on this supposition an explanation is still required for the difference in action between the proteoclastic enzymes. Pepsin does not hydrolyse any of the synthetical polypeptides, trypsin hydrolyses the majority and the other enzymes seem to hydrolyse all of them. The difference in action between trypsin and the other enzymes is probably accounted for by a different arrangement of the units. Fischer supposed that the chain of units in the synthetical polypeptides was not long enough; yet length of chain hardly accounts for the difference in action between pepsin and trypsin on proteins. Pepsin appears to have some special function. So far only simple anhydrides of amino acids have been prepared and their behaviour to enzymes has scarcely been investigated. The presence of anhydride rings in the protein molecule may be the explanation of the difference. Not only will they account for a difference in action, but also they may give a reason for the stability of certain scleroproteins to hydrolysis by trypsin until they have been acted upon by pepsin. We may imagine a protein to be built up as follows :—



This structure represents:—

1. The guanidine group in arginine remaining free in the linking together of amino acids.

2. The second carboxyl group of the dibasic acid combined with ammonia; other amino acids might be attached before the final COOH group is combined with ammonia.

3. Any of the units except proline taking a position in any part of these chains; proline, since it contains only a carboxyl group for linkage, must stand at the end of a chain.

4. A diamino acid continuing the acid amide formation when a proline unit occurs.

5. The possibility of rings being made by salt formation of the free carboxyl group arising from a dibasic acid (either directly or indirectly as under 2) with either the terminal proline or the terminal guanidine group of arginine.

6. A diketopiperazine or anhydride ring formed by the final union of the terminal NH and CO groups.

Such a structure will explain the difference in action between pepsin and trypsin. Trypsin will hydrolyse a chain of amino acids with a terminal amino or carboxyl group. Pepsin will open the anhydride ring at one or more junctions and will give several proteoses and peptones with free terminal NH_2 and COOH groups capable of being attacked by trypsin. Those proteins which are resistant to the action of trypsin until they have been acted upon by pepsin will have all their units contained in the anhydride ring.

The presence of a diamino acid for the continuation of the linking is not probable, because some proteins contain no lysine, and may at the same time contain a large amount of proline. Lysine also apparently stands at the end of a chain since it is not obtained by hydrolysis after the action of nitrous acid, and since after the treatment of protein with alkali it is obtained in the optically active form (Dakin). Kossel has also shown that the ε-amino group of lysine takes no part in the linking of the units in certain protamines.

It is only the presence of proline as unit which insists upon the use of the diamino acid to continue the chain. An anhydride ring need not contain lysine for the attachment of a chain. This need only be attached to a dibasic acid in the ring, and under these conditions the end member of such a chain will have a free carboxyl group which can be neutralised by salt formation with arginine or lysine or by formation of amide with ammonia. It is still very probable that proline is

not a primary product as has often been suggested by Fischer and other observers, and if this be the case the chief argument against the above structure is removed.

The continuation of a chain, should proline be a member, can still proceed if the combination take place between its imino group and a carboxyl group of another amino acid. This has also been suggested by Kossel. We have at present no evidence for the linking of proline in this manner.

THE CONDENSATION PRODUCTS OF AMINO ACIDS.

ANHYDRIDES.

Condensation products of amino acids have been obtained by various investigators by simply heating the amino acids to a temperature of about 200° C.

Leucinimide.—Hesse, in 1857, obtained by distilling a substance, which was called pseudoleucine and was isolated from the products resulting from the putrefaction of yeast, small quantities of a compound termed "leucinsäurenitril". This compound was also obtainable from leucine. A better yield resulted if the compounds were heated in a current of carbon dioxide. Hesse and Limpricht three years later showed that this compound was identical with a crystalline compound isolated by Bopp in 1849 from amongst the products of hydrolysis of caseinogen. Kohler, in 1865, again prepared this compound by heating leucine to 220-230° C. in a current of hydrogen chloride and showed that it lost 20 per cent. by weight in the process. The resulting crystalline mass was insoluble in water, but soluble in hot alcohol from which it crystallised out on cooling. This substance was identical with that of Bopp and of Hesse and Limpricht and from a study of its properties he gave it the more appropriate and correct name of leucinimide.

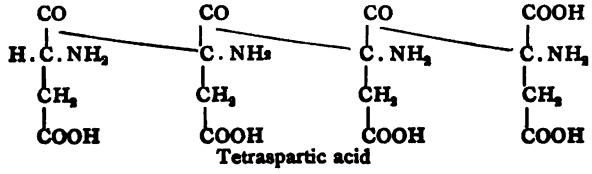
Lactimide.—A similar experiment was made by Preu with alanine and an anhydride—lactimide—of the formula $C_6H_7N_2O_4$ was obtained.

Anhydrides of Aspartic Acid.—Schaal, in 1871, heated asparagine hydrochloride in a current of carbonic acid for three days at 180° C.; he obtained a hard white mass, the greater portion of which was insoluble in water and the remainder soluble only with difficulty. The insoluble body was formed by the loss of fifteen molecules of water from eight molecules of aspartic acid, and the other body by the loss of seven molecules of water from four molecules of aspartic acid:—

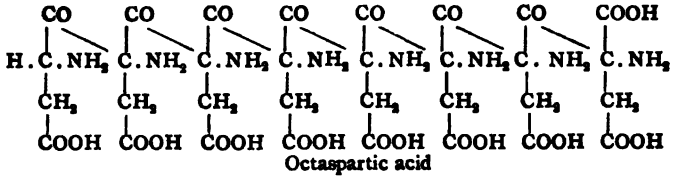
$$C_{16}H_{14}N_4O_9 = 4C_4H_7NO_4 - 7H_2O$$
$$C_{32}H_{26}N_8O_{17} = 8C_4H_7NO_4 - 15H_2O$$

Both compounds were converted into aspartic acid by hydrolysis with baryta water.

J. Guareschi, in 1876, further investigated these substances by determining the amount of silver in the silver salts, but their nature was only demonstrated in 1897-1899 by Schiff. He obtained them by heating aspartic acid, prepared from asparagine and dried at 110° C., for twenty hours at 190-200° C., the yield amounting to 72-75 per cent. Not only were the anhydrides, octaspartide and tetraspartide, as Schiff called these compounds, formed in the process, but also the tetraspartic and octaspartic acids. These acids he also prepared from the anhydrides by hydrolysis with the calculated quantity of cold dilute alkali. From the analysis of their salts, as also their anilides and phenylhydrazides, and from the fact that they gave the biuret reaction which was not observed by Schaal, but pointed out by Grimaux in 1882, he gave these acids the following formulæ:—

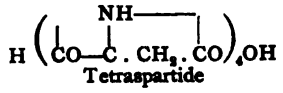


Tetraspartic acid

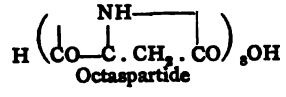


Octaspartic acid

and their anhydrides:—



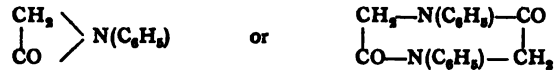
Tetraspartide



Octaspartide

The octaspartic acid was an octobasic acid, its ninth carboxyl group being neutralised by the adjacent NH_2 group.

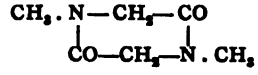
Anhydrides of Phenylglycine and Phenylalanine.—The products obtained by heating phenylglycine in an open vessel to the temperature of 140-150° C. were examined by P. J. Meyer in 1877. An anhydride of the formula :—



was isolated by crystallisation of the crude mass from alcohol and the presence of an intermediate compound was suspected.

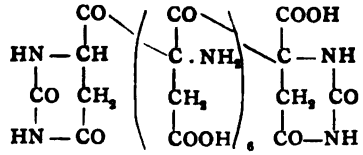
Phenyllactimide was prepared, in 1883, by Erlenmeyer and Lipp by the dry distillation of phenylalanine. This compound had properties analogous to leucinimide and lactimide.

Anhydride of Sarcosine.—The effect of heat upon an amino acid and the exact composition of the resultant product was most carefully studied by Mylius, in 1884, in the case of sarcosine (methylglycine). Sarcosine was found to melt at 210-215° C.; it became quite liquid at 220° C. Water, carbon dioxide and a volatile base (dimethylamine) were evolved, and an oil remained. On distilling the oil a crystalline mass resulted which was recrystallised from alcohol. Analysis and molecular weight determinations showed that the substance had the formula $C_6H_{10}N_2O_2$: on oxidation it gave dimethyloxamide and oxalic acid. It was therefore represented by the structural formula :—



Mylius did not point out the resemblance of his substance to the other anhydrides, and he consequently failed to clear up the nature of the class of amino acid anhydrides. Their structure was only verified by the later work of E. Fischer.

Anhydrides with Urea, etc.—The researches of Schützenberger between 1875 and 1880 upon the products of hydrolysis of proteins by the action of baryta water under pressure, led the French chemists to the belief that the proteins were composed of amino acids and urea or oxamide. In 1882 therefore Grimaux heated Schaal's aspartic acid anhydride with urea for two hours at 125-130° C. A thick mass almost entirely soluble in water resulted; its solution was gelatinous and difficult to filter, and it possessed the properties of colloidal substances, behaving very like albumin. This polyaspartic ureide gave the biuret reaction, and was converted by baryta into carbonic acid, ammonia and aspartic acid; it had the formula $C_{34}H_{40}N_{10}O_{25}$, and consisted of eight molecules of aspartic acid and two molecules of urea. Schiff gave it the formula:—



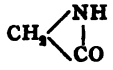
In 1888 Schützenberger, who regarded proteins to be composed of (1) urea and oxamide; (2) leucines, or amino acids of the formula $C_nH_{2n+1}NO_2$, where n = 6, 5, 4, 3, 2; (3) leuceines, or amino acids of the formula $C_nH_{2n-1}NO_2$, where n = 4, 5, 6, and that there was one molecule of leucine to one molecule of leuceine, prepared the leuceines by the action of ethylene dibromide upon the zinc salts of the lower leucines, such as glycine and alanine, according to the equations

$$C_2H_5NO_2 + C_2H_4Br_2 = 2HBr + C_4H_7NO_2$$
$$C_3H_7NO_2 + C_2H_4Br_2 = 2HBr + C_5H_9NO_2$$

and in 1891 he heated a mixture of leucine and leuceine with 10 per cent. of urea, carefully dried at 110° C., with phosphoric anhydride. He obtained a mass soluble in water, which was precipitated by several volumes of alcohol; it gave the biuret reaction and other protein reactions, and Schützenberger regarded it as a "pseudo-peptone synthetique".

Anhydrides of Glycine and the Biuret Base.

In 1883 Curtius first prepared glycine ester by decomposing glycine ester hydrochloride with silver oxide. It was a colourless, strongly basic oil, very unstable, and only capable of preservation in dry ether. If it were allowed to stand in the air, it underwent decomposition and was converted into an insoluble anhydride,

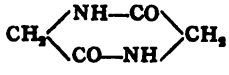


and a soluble base, which gave the biuret reaction and was called the *biuret base.*

Further investigations upon the nature of these compounds were made by Curtius and Goebel in 1888, who found that the glycine anhydride separated when the ester was allowed to stand for a few days with four volumes of water, and that from the analysis of its silver and copper compounds it had the formula

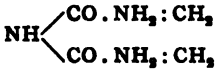
No result could be arrived at concerning the biuret base, which was prepared by keeping pure glycine ester in a sealed tube, when it changed into a white crystalline mass.

Curtius and Schulze, in 1890, by molecular weight determinations found that the formula of the anhydride must be doubled, and it was probably represented by



which was ultimately proved by Fischer and Fourneau in 1901.

The biuret base was investigated again in 1894 by Lilienfeld, who prepared it by heating glycine ester with solid potassium bisulphate on the water bath, and who gave it the formula $C_4H_9N_3O_3$, and the constitution



since he also obtained dimethylamine, ethyl ether and carbonic acid in its preparation.

When he heated it with water, Lilienfeld obtained a flocculent precipitate, such as Curtius and Goebel had observed; this formed a gelatinous mass after filtering which contracted like gelatin and behaved, in fact, very like glutin. In a similar manner Lilienfeld con-

densed leucine ester and tyrosine ester with glycine ester and obtained a peptone-like body giving all the principal protein reactions.

Another anhydride of glycine was obtained in 1900 by Balbiano and Trasciatti who heated glycocoll with glycerol; it was a yellowish powder like the horny substances insoluble in all neutral solvents, and on hydrolysis Balbiano found that it was reconverted into glycine.

Not, however, until 1904 were any further investigations carried out concerning the constitution of the biuret base. Schwarzschild then suggested that it consisted of seven glycine molecules combined together in an open chain, and that it was the ethyl ester of hexaglycyl-glycine of the formula

$$NH_2 . CH_2 . CO—(NH . CH_2 . CO)_5—NH . CH_2 . COOC_2H_5,$$

but Curtius shortly afterwards showed that this was erroneous, and that the body with which Schwarzschild had worked was still a mixture of glycine anhydride and biuret base.

By studying the conditions under which glycine ester was converted into glycine anhydride and biuret base, Curtius showed that, when moisture was excluded as completely as possible, the biuret base with only traces of glycine anhydride was formed, and that the amount of glycine anhydride produced increased with the amount of water present. Thus, if pure glycine ester were kept in the absence of air, it solidified in a few days and the mass contained biuret base with 23-24 per cent. of glycine anhydride; if glycine ester were boiled with dry chloroform, 12 per cent. of glycine anhydride was formed, but if perfectly pure glycine ester were mixed with about a third of its volume of absolute ether and left for some weeks it was almost completely converted into biuret base, only 1 per cent. of glycine anhydride being present. The analysis, molecular weight, properties and reactions of the biuret base showed conclusively that it was triglycyl-glycine ester, *i.e.*, a tetra-glycyl compound having the formula

$$NH_2 . CH_2 . CO—NH . CH_2 . CO—NH . CH_2 . CO—NH . CH_2 . COOC_2H_5.$$

It was observed by Curtius and Gumlich that the biuret base when heated *in vacuo* to 100° C. lost alcohol, and that it was converted into an anhydride, most probably octaglycine anhydride, so that glycine ester forms a starting point from which quite complex substances can be derived. Such substances have also been obtained by Emil Fischer and his pupils by the condensation of amino acid and polypeptide esters, p. 29.

THE LINKING TOGETHER OF AMINO ACIDS.

By the action of benzoyl chloride upon the silver salt of glycine, Curtius, in 1881, obtained in addition to the expected hippuric acid,

$$C_6H_5COCl + H_2N.CH_2.COOH = HCl + C_6H_5CO—NH.CH_2.COOH,$$

two other acids of higher molecular weight. One of these was hippuryl-glycine or benzoyl-glycyl-glycine,

$$C_6H_5.CO—HN.CH_2.CO—NH.CH_2.COOH,$$

as was proved by the study of its salts, its ethyl ester and amide and by its hydrolysis into hippuric acid and glycine. It was the first definite compound known which contained two amino acid residues combined together.

The constitution of the other acid, called the γ-acid, could not be determined; it had the formula $C_{10}H_{12}N_2O_4$, was soluble with difficulty in water and gave the biuret reaction. It was obtained in larger quantities when hippuric ester was fused with glycine, but under these conditions another compound—benzoyl-bisglycyl-glycine—was formed at the same time, so that in this way a series of compounds resulted, each succeeding member containing a glycyl —$NH_2.CH_2.CO$— group more than the preceding one.

Further investigations upon the constitution of these compounds were only carried out in 1904 by Curtius and Benrath, who found that the γ-acid from the analysis of its silver salt, ester, etc., had the formula $C_{19}H_{24}N_6O_8$, and that it was benzoyl-pentaglycyl-glycine,

$$C_6H_5.CO—(NH.CH_2.CO)_5—NH.CH_2.COOH,$$

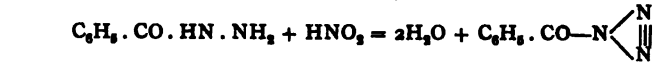
its ester being identical with the compound synthesised by Curtius and Wüstenfeld (see below).

Two other compounds—hippuryl-glycine and benzoyl-triglycyl-glycine—were found to be formed by fusing together hippuric ester and glycine, but not the previously isolated benzoyl-bisglycyl-glycine. Longer chains than the six membered γ-acid are not believed by Curtius and Benrath to be formed in this reaction.

In 1890 Curtius, by the action of hydrazine upon benzoyl chloride, benzamide or benzoic ester, obtained benzoylhydrazine,

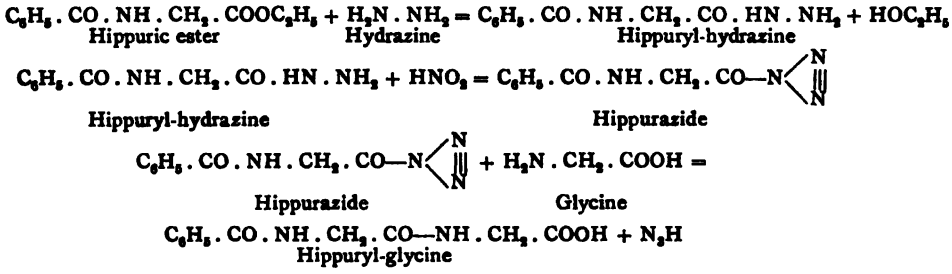
$$C_6H_5.COOC_2H_5 + H_2N.NH_2 = C_6H_5.CO.HN.NH_2 + HOC_2H_5,$$

which, when treated with nitrous acid, gave benzoylazoimide,

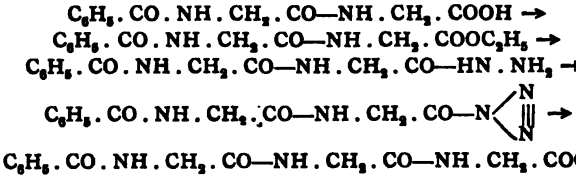


By combining benzoylazoimide with glycine, he synthesised hippuric acid,

and by carrying out the same series of reactions with hippuric acid, the compound hippurazide or hippurylazoimide was made, which could be employed in synthesis in the place of the unknown hippuryl chloride; thus from hippuric ester by the following series of reactions hippuryl-glycine was obtained identical with the compound previously obtained in 1883 by the action of benzoyl chloride upon the silver salt of glycine:—



This method of combining together amino acids was further extended by Curtius and Wüstenfeld in 1902 by preparing the ester, the hydrazide and azide of this compound and again combining it with glycine, when benzoyl-diglycyl-glycine was obtained:—



From this compound benzoyl-triglycyl-glycine was prepared by the same series of reactions, and benzoyl-tetraglycyl-glycine by continuing the process.

Further experiments upon the formation of glycyl chains with hippurazide were carried out by Curtius and Levy; by combining hippurazide with glycyl-glycine ester, prepared by the method of Fischer and Fourneau (see below), benzoyl-diglycyl-glycine ester, identical with the compound prepared by Curtius and Wüstenfeld, was obtained. The

azide of this compound when combined with glycyl-glycine hydrochloride gave benzoyl-tetraglycyl-glycine :—

$$C_6H_5 . CO—(NH . CH_2 . CO)_4—NH . CH_2 . COOH.$$

This compound was also prepared from benzoyl-triglycyl-glycine azide and glycine ester.

The further lengthening of the chain by means of the azide of benzoyl-tetraglycyl-glycine could not be accomplished since this compound could not be prepared, but the next member of the series, benzoyl-pentaglycyl-glycine ester,

$$C_6H_5 . CO—(NH . CH_2 . CO)_5—NH . CH_2 . COOC_2H_5,$$

was prepared from benzoyl-triglycyl-glycine azide and glycyl-glycine ester. This was identical with the original γ-acid of 1883, of which Curtius and Benrath had determined the constitution.

By condensing the biuret base, which in the meanwhile had been proved to be triglycyl-glycine ester with hippurazide, Curtius and Levy again obtained the former benzoyl-tetraglycyl-glycine, and by condensing it with hippuryl-glycine azide they obtained benzoyl-pentaglycyl-glycine ester, and thus by a less circuitous method attained to the same compound as they had prepared from hippurazide. Further lengthening of the glycyl chain has not as yet been carried out by this method, but the method has been adapted by Curtius and Lambotte to the formation of alanine chains, namely :—

Hippuryl-alanine, $C_6H_5 . CO . NH . CH_2 . CO—NH . CH(CH_3) . COOH$ from hippurazide and α-alanine.

Hippuryl-alanyl-alanine, $C_6H_5 . CO . NH . CH_2 . CO — NH . CH(CH_3) . CO — NH . CH(CH_3) . COOH$ from hippuryl-alanine azide and α-alanine.

Hippuryl-alanyl-alanyl-alanine, $C_6H_5 . CO . NH . CH_2 . CO — NH . CH(CH_3) . CO — NH . CH . (CH_3) . CO—NH . CH(CH_3) . COOH$ from hippuryl-alanyl-alanine azide and α-alanine.

These all contain the glycine residue as well as the alanine residue in their molecule; in order to eliminate the glycine residue and obtain compounds without the glycine radicle, Curtius and van der Linden prepared the following compound :—

Benzoyl alanyl-alanine, $C_6H_5 . CO . NH . CH(CH_3) . CO — NH . CH(CH_3) . COOH$ from benzoyl-alanine azide, which was prepared from benzoyl alanine, as obtained by Fischer's method, and alanine.

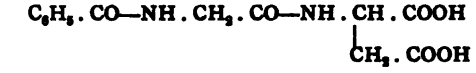
Benzoyl alanine azide was also combined with glycine, and this radicle introduced at the end of the chain; thus they prepared

Benzoyl alanyl-glycine, $C_6H_5 . CO . NH . CH(CH_3) . CO—NH . CH_2 . COOH$ from benzoyl-alanine azide and glycine.

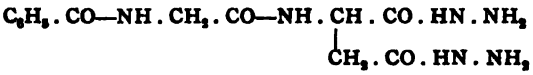
Benzoyl alanyl-glycyl-glycine, $C_6H_5 . CO . NH . CH(CH_3) . CO — NH . CH_2 . CO — NH . CH_2 . COOH$ from benzoylalanyl-glycine azide and glycine, or benzoyl alanine azide and glycyl-glycine.

The behaviour of hippurazide with the dibasic aspartic acid was

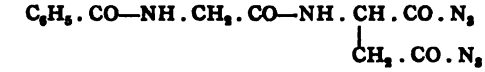
investigated by Th. and H. Curtius in order to build up chains containing this amino acid. By the action of hippurazide upon aspartic acid in alkaline solution they obtained hippuryl-aspartic acid:—



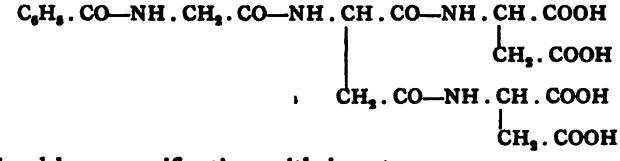
The ester of this compound was converted into the hydrazide by means of hydrazine,



from which hippuryl-aspartic acid azide was obtained by the action of nitrous acid:—

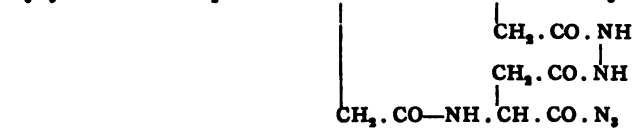


This reacted in ethereal solution with aspartic ester yielding hippuryl-asparagyl-aspartic ester from which the free acid,

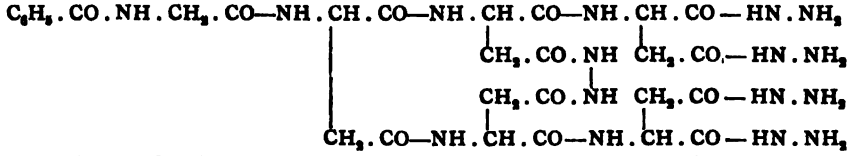


was obtained by saponification with baryta.

The hydrazide of this compound was then prepared from the ester in the usual manner, and from this the azide, which did not, however, possess the normal structure, but that of the hydrazi-azide,

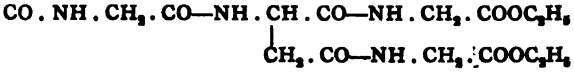


The condensation product of this compound with aspartic ester was not isolated, but the complex hippuryl-disaspartyl-aspartic acid hydrazi-hydrazide, its hydrazine derivative,



was obtained when the condensation product was treated in alcoholic solution with hydrazine hydrate.

Just in the same way hippuryl-aspartyl-glycine ester,



resulted when hippuryl-aspartic acid azide was combined with glycine ester.

In conjunction with Gumlich, Curtius has investigated the linking of hippurazide with β-amino-α-oxypropionic acid and with β-amino-butyric acid. With the former compound the combination took place with the hydroxyl group instead of with the amino group, hippuryl-α-oxy-β-aminopropionic acid,

$$C_6H_5 . CO . NH . CH_2 . CO—O—CH . (CH_2 . NH_2) . COOH,$$

being formed. With the latter compound by the usual series of reactions they prepared hippuryl-β-aminobutyric acid and hippuryl-β-aminobutyryl-β-aminobutyric acid.

Curtius and Müller have also prepared hippuryl-γ-aminobutyric acid and hippuryl-β-phenyl-α-alanine, compounds of no great interest since these amino acids do not occur in the protein molecule. They show, however, that not only can α-amino acids be combined together by the azide method, but also β- and γ- substituted amino acids.

In order to build up chains containing the carbamic acid radicle, NH . CO, just as Curtius and his co-workers have built up chains containing glycyl, alanyl and asparagyl radicles, Curtius and Lenhard, in 1904, proposed to make use of the azide of hippuryl-carbamic acid,

$$C_6H_5 . CO . NH . CH_2 . CO—NH . CO . N_3.$$

This compound was not available, since sufficient quantities of hippuryl urea, which Curtius had formerly prepared from hippuric ester and urea, could not be obtained from hippurazide and urea. They therefore attempted to make the azide of benzoylcarbamic acid by the action of hydrazine on benzoylurea, but the only product which they obtained was the hydrazide of benzoic acid. The benzoic acid radicle is therefore very easily eliminated from the urea molecule, the molecule of benzoylcarbamic acid hydrazide being hydrolysed according to the equation :—

$$C_6H_5 . CO . NH . CO . NH . NH_2 + H_2O = C_6H_5 . CO . NH . NH_2 + NH_3 + CO_2.$$

This non-success led them to attempt to combine phenylcarbamic acid azide $C_6H_5 . NH . CO—N_3$, which Curtius and Hofmann and Curtius and Burkhardt had described in 1896 and 1898, with urea, but again the desired result was not achieved, nor could a combination of this compound with biuret be effected.

It followed therefore that acid radicles cannot be combined with urea by the acid azide reaction.

When glycine was used instead of urea for combination with the

azide of phenylcarbamic acid phenylcarbaminoglycine resulted, which was identical with the compound prepared by Paal in 1894 from phenylisocyanate and glycine.

With this compound Curtius and Lenhard continued the lengthening of the chain by the azide reaction, and obtained

Phenylcarbaminoglycyl-glycine, $C_6H_5 . NH . CO—NH . CH_2 . CO—NH . CH_2 . COOH$, and phenylcarbaminodiglycyl-glycine, $C_6H_5 . NH . CO—NH . CH_2 . CO—NH . CH_2 . CO—NH . CH_2 . COOH$.

These compounds and their various derivatives prepared by Curtius and his pupils are white crystalline compounds, most of which are soluble with difficulty in cold water. Some of them give the biuret reaction, but others do not, in particular the less complex compounds where the influence of the acid radicle inhibits the reaction, although the compounds possess the exact conditions, as determined by Schiff, for the positive exhibition of the reaction.

The reactions given by the azides with alcohol, ammonia, aniline, etc., are of greater interest and may therefore be briefly summarised.

By the action of ammonia the acid azides are either completely saponified into the corresponding acids, or they are converted into derivatives of urea by a rearrangement in the molecule. With the dibasic acids both possibilities may occur at the same time, and the resulting compound is half acid amide and half urethane. Subsequent hydrolysis clearly shows the nature of the component amino acid chain. Thus, hippuryl urethane is formed from hippurazide and alcohol,

$$C_6H_5 . CO . NH . CH_2 . CO . N_3 + C_2H_5OH = C_6H_5 . CO . NH . CH_2 . NH . COOC_2H_5 + N_2,$$

and on hydrolysis it is converted into benzoic acid, ammonia, carbonic acid and formaldehyde :—

$$C_6H_5 . CO . NH . CH_2 . NH . COOC_2H_5 + 3H_2O = C_6H_5 . COOH + 2NH_3 + HCHO + CO_2 + C_2H_5OH.$$

The reaction therefore leads to the formation of formaldehyde from glycine.

Hippuryl alanine azide and aniline give the following urea derivative :—

$$C_6H_5 . CO . NH . CH_2 . CO—NH . CH(CH_3) . CO—NH . C_6H_5,$$

which, on hydrolysis, breaks down into hippuric acid, ammonia, acetaldehyde, carbonic acid and aniline :—

$$C_6H_5 . CO . NH . CH_2 . CO—NH . CH(CH_3) . CO—NH . C_6H_5 + 3H_2O = C_6H_5 . CO . NH . CH_2 . COOH + 2NH_3 + CH_3 . CHO + CO_2 + H_2N . C_6H_5.$$

The same products are obtained when the urethane derivative, obtained from benzoylalanine azide and alcohol, is hydrolysed :—

$$C_6H_5 . CO . NH . CH . (CH_3) . NH . COOC_2H_5 + 3H_2O = C_6H_5 . COOH + 2NH_3 + CH_3CHO + CO_2 + C_2H_5OH,$$

except that benzoic acid appears in the place of hippuric acid. Hippuryl aspartic acid azide and aniline give a compound which is half anilide and half carbanilide, and this on hydrolysis is converted into α-β-diaminopropionic acid, hippuric acid, aniline and carbonic acid :—

$$C_6H_5.CO.NH.CH_2.CO{-}NH.\underset{\displaystyle CH_2.NH.CO.NH.C_6H_5}{\overset{\displaystyle |}{CH}}.NH.CO.NH.C_6H_5 + 3H_2O =$$
$$C_6H_5.CO.NH.CH_2.COOH + \underset{\displaystyle CH_2.NH_2}{\overset{\displaystyle |}{NH_2.CH.COOH}} + 2C_6H_5.NH_2 + CO_2$$

The normal urethane formed by the action of alcohol on hippuryl aspartic acid azide yields on hydrolysis hippuric acid, carbonic acid, alcohol and aminoacetaldehyde :—

$$C_6H_5.CO.NH.CH_2.CO{-}NH.\underset{\displaystyle CH_2.NH.COOC_2H_5}{\overset{\displaystyle |}{CH}}.NH.COOC_2H_5 + 4H_2O =$$
$$C_6H_5.CO.NH.CH_2.COOH + 2NH_2 + NH_2.CH_2.CHO + CO_2 + 2C_2H_5OH$$

The first reaction shows the conversion of a compound belonging to the series of dibasic monoamino acids into a diaminomonocarboxylic acid; in the second reaction a dibasic amino acid is changed into the aldehyde of the monobasic glycine.

Finally, propylenediamine was obtained when the urethane, resulting from the action of alcohol upon hippuryl-β-aminobutyric acid azide, was hydrolysed :—

$$C_6H_5.CO.NH.CH_2.CO{-}NH.CH(CH_3).CH_2.NH.COOC_2H_5 + 3H_2O =$$
$$C_6H_5.COOH + NH_2.CH_2.COOH + NH_2.CH(CH_3).CH_2.NH_2 + CO_2 + C_2H_5OH$$

which shows the conversion of an amino acid derivative into a diacid base.

The further work of Curtius and his pupils, published in 1906, is concerned with the action of nitrous acid upon the polyglycine compounds: diazoacetyl glycine ester $N_2:CH.CO{-}NH.CH_2.COOC_2H_5$ was formed by the action of nitrous acid upon glycyl-glycine ester hydrochloride and diazoacetyl glycyl-glycine ester,

$$N_2:CH.CO{-}NH.CH_2.CO{-}NH.CH_2.COOC_2H_5,$$

by its action upon diglycyl-glycine ester hydrochloride, and similarly diazoacetyl-bisglycyl-glycine ester.

By the action of ammonia on the first body diazoacetyl glycine amide,

$$N_2:CH.CO{-}NH.CH_2.CO.NH_2,$$

was formed, and on the latter diazoacetyl glycyl-glycine amide,

$$N_2:CH.CO{-}NH.CH_2.CO{-}NH.CH_2.CO{-}NH_2.$$

These are yellow substances, but when excess of ammonium hydroxide or liquid ammonia is added to the esters colourless substances are

obtained; they were at first regarded as azomethane derivatives, but have since been shown to be the ammonium salts, *e.g.*, of isodiazoacetyl glycine amide,

The later work of Curtius and his pupils is upon the action of hydrazine on these compounds: diazoacetic ester did not give the expected hydrazide, but lost ammonia and yielded the hydrazide of triazo-acetic acid. Diazoacetyl glycine ester gave the hydrazide, which was converted on further action into the diammonium salt of 5-oxytriazole-1-acetic hydrazide. The next homologue in the series behaved like diazoacetyl glycine ester. Hydrochloric acid gas converted diazoacetylglycine hydrazide into chloracetyl-glycine hydrazide hydrochloride; chloracetyl-glycine-azide resulted on treatment with nitrous acid.

These compounds have given us an insight into complex glycine, alanine and aspartic acid derivatives. Since E. Fischer has prepared by his methods (see under polypeptides) compounds containing these amino acids without the presence of the benzoyl group which is strange to the protein molecule, no great interest has been attached to them, but at present the aspartic acid compounds, if we disregard Schiff's polyaspartic acid, which probably has another constitution than that represented, are the most complex substances known containing this important constituent of the protein molecule.

The transformations of the derivatives increase our interest in these compounds prepared by Curtius and his pupils, and give an impulse to their further study, especially as formaldehyde is such an important compound in the synthesis of sugars by plants, and as the diamino acids and diamines occur as products of decomposition of proteins by enzymes and bacteria, although according to our present knowledge they are not formed in nature in this manner.

THE POLYPEPTIDES.

Synthesis.

The three methods which have been devised by Fischer for the synthesis of the polypeptides have been mentioned in the introduction. A description of all the polypeptides which have been prepared is impossible, but Tables have been compiled giving the compounds obtained by the various methods. A fuller account of the methods is also necessary for the proper comprehension of the enormous work which has been expended on the synthesis of the higher polypeptides.

Method I. By Formation and Hydrolysis of the Anhydrides (Diketopiperazines).

It had been found by previous investigators that leucine, alanine and other amino acids were converted on heating into anhydrides. Curtius and Goebel, in 1888, showed that glycine ester was decomposed by water, or by distillation, and was converted with loss of alcohol into an anhydride which probably had the formula :—

$$\begin{array}{l} NH—CH_2—CO \\ | \qquad\quad | \\ CO—CH_2—NH \end{array}$$

These anhydrides form the starting-point in the synthesis of the polypeptides by this method, and they are best obtained by heating the ester of the amino acids in a sealed tube to 150-180° C. for some hours.

Fischer and Fourneau, in 1901, found that glycine anhydride was converted by boiling with concentrated hydrochloric acid into the hydrochloride of an amino acid of the formula $C_4H_8N_2O_3$; from this they obtained the free acid by treatment with the calculated quantity of caustic soda, or by means of silver oxide, and represented its formation by the equation :—

$$\begin{array}{l} NH—CH_2—CO \\ | \qquad\quad | \\ CO—CH_2—NH \end{array} + H_2O = NH_2.CH_2.CO—NH.CH_2.COOH.$$

The new compound is the first anhydride of glycine, and it was termed glycyl-glycine, the group $NH_2.CH_2.CO$ being called the glycyl group.

Glycyl-glycine ester resulted when glycine anhydride was treated with alcoholic hydrochloric acid :—

$$\begin{array}{l} NH—CH_2—CO \\ | \qquad\quad | \\ CO—CH_2—NH \end{array} + C_2H_5OH = NH_2.CH_2.CO—NH.CH_2.COOC_2H_5.$$

Both the free acid and its ester have a great tendency to become reconverted into glycine anhydride, and both compounds are characterised by the great reactiveness of the NH_2 group; thus, with phenylisocyanate they both yield the compound

$$C_6H_5.NH.CO.NH.CH_2.CO.NH.CH_2.COOH;$$

and the ester on combination with ethyl chlorocarbonate gives carbethoxyl-glycyl-glycine ester:—

$$Cl.CO.OC_2H_5 + NH_2.CH_2.CO.NH.CH_2.COOC_2H_5 =$$
$$HCl + C_2H_5O.OC.NH.CH_2.CO—NH.CH_2.COOC_2H_5,$$

from which the amide,

$$C_2H_5O.OC.NH.CH_2.CO—NH.CH_2.CONH_2,$$

is obtained by the action of ammonia, and the free acid,

$$C_2H_5O.OC.NH.CH_2—CO.NH.CH_2.COOH,$$

by careful hydrolysis with soda.

When carbethoxyl-glycyl-glycine ester was heated with leucine ester combination occurred and the compound, carbethoxyl-glycyl-glycyl-leucine ester, was formed:—

$$C_2H_5O.OC.NH.CH_2.CO.NH.CH_2.COOC_2H_5 + NH_2.CH(C_4H_9).COOC_2H_5 =$$
$$C_2H_5OH + C_2H_5O.OC.NH.CH_2.CO.NH.CH_2.CO.NH.CH(C_4H_9).COOC_2H_5.$$

This compound contains three amino acids combined together and was the first known representative of a tripeptide.

Carbethoxyl-glycyl-glycine amide and carbethoxyl-glycyl-glycyl-leucine ester give the biuret reaction as would be expected from the researches of Schiff in 1900, who found that glycine amide $NH_2.CH_2.CO.NH_2$ also gave the reaction.

In the same way alanyl-alanine and alanyl-alanine ester, which yielded carbethoxyl-alanyl-alanine ester when treated with ethylchlorocarbonate, were obtained from alanine anhydride, and leucyl-leucine from leucine anhydride.

The hydrolysis of the anhydrides of the higher amino acids is more difficult to effect. The diketopiperazine ring is not easily split open by means of acid, and although Fischer, in 1905, discovered that the diketopiperazine could be easily converted into the dipeptide by treatment with the equimolecular quantity of caustic soda in 10-15 minutes at the ordinary temperature in the cases of glycyl-glycine and alanyl-alanine, yet in other cases, such as that of leucine anhydride, the anhydride was very resistant to alkali; in this case hydrolysis was best effected by hydrobromic acid. It appears that the stability of the diketopiperazine ring is connected with the nature of

the alkyl groups attached to it, and that there is here another instance of steric hindrance.

The dipeptides of the oxy- and diamino acids have so far only been prepared by this method by Fischer and Suzuki; here, the methyl esters of the amino acids were found to be most easily converted into the anhydrides, and hydrolysis by alkali proceeded readily.

Several of the dipeptides are most easily prepared in this way, and they have been employed in the synthesis of more complex polypeptides. The method, however, does not lend itself to the preparation of higher polypeptides, but it will be observed that pentaglycyl-glycine and another compound, probably octaglycine anhydride, have been prepared by heating the methyl ester of diglycyl-glycine. The various compounds isolated by Curtius and his pupils, such as glycine anhydride and the biuret base, have been obtained from glycine ester.

It must be noted that anhydrides are also formed when the dipeptides, prepared by the other methods, are heated to their melting-points, *e.g.*, leucyl-alanine anhydride from leucyl-alanine.

Mixed anhydrides, as for example glycyl-alanine anhydride, cannot be obtained by heating a mixture of the esters, since a complex mixture would result, but they are easily prepared by the action of ammonia upon the esters of the dipeptides obtained by the other methods. The same anhydride is formed from the two corresponding dipeptides as was first proved in the case of leucyl-glycine ester and glycyl-leucine ester which gave leucyl-glycine anhydride:—

$$C_4H_9 . CH . (NH_2) . CO—NH . CH_2 . COOC_2H_5 = C_4H_9 . \underset{\displaystyle NH—CO—CH_2}{\overset{\displaystyle CH—CO—NH}{|\qquad\qquad|}} + C_2H_5OH$$

$$H_2N . CH_2 . CO—NH . CH(C_4H_9) . COOC_2H_5 = \underset{\displaystyle CO—CH(C_4H_9)—NH}{\overset{\displaystyle HN — CH_2 — CO}{|\qquad\qquad|}} + C_2H_5OH$$

These compounds, of which several have now been prepared, are of great importance as they serve for the isolation of dipeptides from a mixture of polypeptides and amino acids (see p. 81). On hydrolysis they yield a mixture of the two dipeptides, which are composed of the amino acids of which they are built up. Thus glycyl-l-tyrosine anhydride yielded glycyl-l-tyrosine and l-tyrosyl-glycine. The latter compound is the first example of a polypeptide containing the tyrosine radicle as the acyl group; in all the other polypeptides in which tyrosine is present it stands at the end of the chain.

The compounds which have been obtained can be seen from the accompanying list.

POLYPEPTIDES SYNTHESISED BY METHOD I.

Simple Polypeptides.

Glycine ester → glycine anhydride → glycyl-glycine.
Alanine ester → alanine anhydride → alanyl-alanine.
Leucine ester → leucine anhydride → leucyl-leucine.
Diaminopropionic acid ester → diaminopropionic acid anhydride.
Histidine ester → histidine anhydride → histidyl-histidine.
Lysine ester → lysine anhydride → lysyl-lysine.
Arginine ester → arginyl-arginine (?)
Serine ester → serine anhydride → seryl-serine.
Isoserine ester → isoseryl-isoserine.
Tyrosine methyl ester → tyrosine anhydride → tyrosyl-tyrosine.
Aspartic acid methyl ester → aspartic acid anhydride → diketopiperazine diacetic diamide by action of ammonia.
2-5-diketopiperazine, 3-6-diacetic acid
Diglycyl-glycine methyl ester → pentaglycyl-glycine ester → pentaglycyl-glycine.
→ octaglycine anhydride (?)
l-Alanyl-glycyl-glycine methyl ester → l-alanyl-diglycyl-l-alanyl-glycyl-glycine.
leucyl-alanine anhydride } from dipeptide.
leucyl-proline anhydride }

Mixed Polypeptides.

Chloracetyl alanine ester → glycyl-alanine anhydride → glycyl-alanine. / → alanyl-glycine.
Chloracetyl tyrosine ester → glycyl-l-tyrosine anhydride → glycyl-l-tyrosine. / → l-tyrosyl-glycine.
Glycyl-l-phenylalanine ester / l-Phenylalanyl-glycine ester → glycyl-l-phenylalanine anhydride
Leucyl-glycine ester → leucyl-glycine anhydride → leucyl-glycine. / → glycyl-leucine.
Glycyl-aspartic acid ester → glycyl-aspartic acid anhydride
leucyl-alanine anhydride → leucyl-alanine. / → alanyl-leucine.
Phenylalanyl-glycine ester → phenylalanyl-glycine anhydride.
Valyl-glycine ester → valyl-glycine anhydride.
Valyl-alanine ester → valyl-alanine anhydride.
l-Valyl-d-valine ester → *trans*-valine anhydride.
Iodopropionyl-l-tryptophane methyl ester → alanyl-tryptophane anhydride.
Glycyl-l-isoleucine-methyl ester → glycyl-l-isoleucine anhydride.
l-Leucyl-d-isoleucine methyl ester → l-leucyl-d-isoleucine anhydride.
Glycyl-aminostearic acid ester → glycyl-aminostearic acid anhydride.
Leucyl-β-aminobutyric acid ester → leucyl-β-aminobutyric acid anhydride.
Glycyl-d-valine methyl ester → glycyl-d-valine anhydride.
d-Alanyl-d-valine methyl ester → d-alanyl-d-valine anhydride.
l-Leucyl-d-valine methyl ester → l-leucyl-d-valine anhydride.
Prolyl-glycine ester → prolyl-glycine anhydride.
Prolyl-leucine ester → prolyl-leucine anhydride.
Glycyl-serine methyl ester → glycyl-serine anhydride.
Alanyl-serine methyl ester → alanyl-serine anhydride.
Glycyl-aminobutyric acid methyl ester → glycyl-aminobutyric acid anhydride.

Method II. By Means of the Halogen Acyl Compounds.

E. Fischer and E. Otto first described this method of synthesising polypeptides in 1903. Just as an ordinary acyl radicle can be combined with an amino acid, *e.g.*, in the preparation of benzoylalanine, so also can a halogen substituted acyl radicle be combined with an amino acid. The subsequent action of ammonia upon this compound replaces the halogen atom by the amino group and a dipeptide results, thus :—

Chloracetyl chloride and alanine yield chloracetyl alanine,

$$Cl.CH_2.COCl + NH_2.CH(CH_3)COOH = Cl.CH_2.CO-NH.CH(CH_3)COOH + HCl,$$

from which, by the action of ammonia, glycyl-alanine is obtained :—

$$Cl.CH_2.CO-NH.CH(CH_3).COOH + 2NH_3 =$$
$$NH_2.CH_2.CO-NH.CH(CH_3)COOH + NH_4Cl.$$

In practice this reaction can be carried out in two ways :—

1. By the action of the halogen acyl chloride upon the alkaline solution of the amino acid. This reaction proceeds well with the higher acyl chlorides which are not rapidly acted upon by water, but with the lower acyl chlorides it must be carried out at a very low temperature, and the yields even then are in many cases very poor.

2. By the action of the halogen acyl chloride upon the ester of the amino acid in anhydrous solvents, such as ether, chloroform, petroleum ether. In this reaction two molecules of amino acid ester are required for one molecule of halogen acyl chloride, since half the ester is removed from the reaction as ester hydrochloride. In order to prevent this, the reaction may be carried out in the presence of alkali or alkali carbonate. Subsequent saponification of the ester follows this operation, and loss results by the action of alkali on the halogen acyl radicle. This method is only used when the reaction gives bad yields in aqueous solution.

Several halogen acyl chlorides are necessary for introducing the various amino acid radicles. These are :—

Chloracetyl-chloride for the introduction of the glycyl radicle.
α-Bromopropionyl-chloride for the introduction of the alanyl radicle.
l-α-Bromopropionyl-chloride for the introduction of the d-alanyl radicle.
α-Bromobutyryl-chloride for the introduction of the α-aminobutyryl radicle.
α-Bromisocapronyl-chloride for the introduction of the leucyl radicle.
d-α-Bromo-β-methyl-β-ethyl propionyl chloride for the introduction of the d-isoleucyl radicle (Abderhalden, Hirsch and Schuler).
α-Bromophenylacetyl-chloride for the introduction of the phenylglycyl radicle.
α-Bromo-hydrocinnamyl-chloride for the introduction of the phenylalanyl radicle.
Phenyl-bromopropionyl-chloride for the introduction of the phenylalanyl radicle.

α-δ-Dibromovaleryl-chloride for the introduction of the prolyl radicle.
Fumaryl-chloride for the introduction of the asparagyl radicle.

In order to introduce the asparagyl group into an amino acid, chloro-succinyl chloride, the corresponding halogen-acyl chloride, cannot be employed, since on treatment with ammonia it yields fumaryl derivatives. These, however, when heated with strong ammonia again take up ammonia forming the asparagyl compound, and hence can be employed for this purpose.

Iodoacetyl-chloride and iodopropionyl-chloride were used by Abderhalden and Guggenheim to prepare glycyl-3, 5-diiodo-l-tyrosine and alanine-3, 5-diiodo-l-tyrosine. Abderhalden and Baumann employed them for combination with tryptophane; these products were not obtained in a crystalline state.

The introduction of the prolyl group into an amino acid by means of *α-δ*-dibromovaleric acid chloride reminds us of the synthesis of proline, in which when the compound is treated with ammonia in order to exchange the Br atoms for NH_2, ammonia is lost and ring formation occurs. Prolyl-alanine is prepared as follows:—

$$CH_2Br.CH_2.CH_2.CHBr.COCl + NH_2.CH(CH_3).COOH$$
$$= HCl + CH_2Br.CH_2.CH_2.CHBr.CO-NH.CH(CH_3)COOH$$
$$CH_2Br.CH_2.CH_2.CHBr.CO-NH.CH(CH_3).COOH + 3NH_3$$
$$= 2NH_4Br + CH_2.CH_2.CH_2.CH.CO-NH.CH(CH_3)COOH$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad NH$$

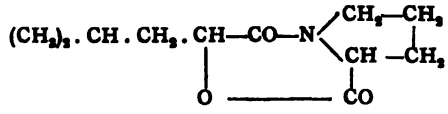
These radicles can be introduced into all the simple mono-amino acids, such as alanine, leucine, tyrosine, etc. ; also into cystine and the dicarboxylic acids when the compounds such as dialanyl-cystine and asparagyl-dialanine are formed. Abderhalden, in conjunction with Guggenheim and Hirszowski, has prepared dipeptides of diiodotyrosine. These can also be prepared by the action of iodine on the corresponding polypeptide containing tyrosine.

Glycyl-amino-acetal was obtained by E. Fischer in 1908 by combining chloracetyl chloride with aminoacetal and subsequent treatment with ammonia. Hydrolysis of the compound by acid gave a product which was most probably glycylaminoaldehyde, *i.e.*, the aldehyde of glycyl-glycine. The combination has also been effected with higher amino acids and *β*-amino acids.

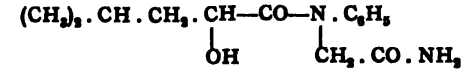
The halogen acyl radicle when combined with proline does not yield the dipeptide on treatment with ammonia. The compound which is formed is apparently the amide of the hydroxy acid, *e.g.*, oxyisocapronyl-proline amide :—

$$(CH_3)_2.CH.CH_2.CH(OH)-CO-N\begin{cases}CH_2-CH_2\\ CH-CH_2\\ CO.NH_2\end{cases}$$

and the anhydride is the lactone :—

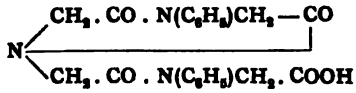


This reaction is apparently due to the tertiary nitrogen atom, for it occurs also with bromisocapronyl-N-phenyl-glycine; the amide,



was formed.

A different reaction took place with chloracetyl-N-phenyl-glycine: the anhydride of imino-diacetyl-N-phenyl-glycine (cf. p. 58) resulted :—



Not only can these radicles be combined with amino acids but also with di, tripeptides, etc., as can be seen from the appended list of polypeptides synthesised by this method. This method only allows of the chain of amino acids being lengthened on one side, namely at the amino group end.

The majority of the polypeptides synthesised by this method are optically inactive, but the optically active compounds can also be prepared by employing the optically active halogen acyl chloride. As previously described under the optically active amino acids, these compounds undergo the Walden inversion; the method therefore allows of the whole of an inactive amino acid being employed for the synthesis of an optically active polypeptide; thus dl-leucine after separation into d-leucine and l-leucine can be converted into l-leucyl-l-leucine by preparing the d-bromisocapronyl chloride from the d-leucine and combining it with l-leucine; treatment with ammonia gives l-leucyl-l-leucine as the compound undergoes the Walden inversion. The four isomers

d-leucyl-l-leucine, l-leucyl-d-leucine } A d-leucyl-d-leucine, l-leucyl-l-leucine } B

can be thus prepared. The A compound is the former inactive leucyl-leucine. Also l-phenylalanyl-glycine was obtained from d-phenyl-*a*-bromopropionyl chloride and glycine, and glycyl-l-phenylalanine from chloracetyl chloride and l-phenylalanine, the dl-phenylalanine used having been separated into its optical isomers by means of its formyl compound.

Methylated polypeptides are obtained by treating the halogen

derivative with methylamine or dimethylamine; the reaction with the tertiary amine leads to the formation of the corresponding hydroxy derivative of the amino acid. Abderhalden and Kautzsch have, however, obtained the trimethyl derivatives by the direct methylation of the dipeptides, leucyl-glycine and glycyl-glycine. Treatment of chloracetyl glycyl-glycine with trimethylamine yielded trimethyl-diglycyl-glycine. Both compounds were isolated as their betaines.

POLYPEPTIDES SYNTHESISED BY METHOD II.

Dipeptides.

Optically Inactive.

Glycyl-alanine.
Glycyl-aminobutyric acid.
Glycyl-phenylalanine.
Glycyl-p-iodophenylalanine.
Glycyl-leucine.
Glycyl-isoleucine.
Glycyl-asparagine.
Glycyl-α-aminostearic acid.
Glycyl-glutamic acid.
Glycyl-serine.
Alanyl-glycine.
Alanyl-alanine.
Alanyl-leucine A.
Alanyl-leucine B.
Alanyl-phenylalanine.
Alanyl-3, 5-diiodo-l-tyrosine.
Alanyl-β-aminobutyric acid.
Alanyl-serine.
α-Aminobutyryl-glycine.
α-Aminobutyryl-aminobutyric acid A.
α-Aminobutyryl-aminobutyric acid B.
Valyl-glycine.
Valyl-alanine.
Leucyl-glycine.
Methyl-leucyl-glycine.
Dimethyl-leucyl-glycine.
Leucyl-alanine.
Leucyl-leucine A.
Leucyl-leucine B.
Leucyl-isoleucine.
Leucyl-phenylalanine α.
Leucyl-phenylalanine β.
Leucyl-isoserine A.
Leucyl-isoserine B.
Leucyl-α-methyl-isoserine A.
Leucyl-α-methyl-isoserine B.
Leucyl-asparagine.
Leucyl-aspartic acid.
Leucyl-β-aminobutyric acid.
Phenylglycyl-glycine.
Phenylglycyl-alanine A.
Phenylglycyl-alanine B.
Phenylglycyl-asparagine.
Phenylalanyl-glycine.
Phenylalanyl-alanine.
Phenylalanyl-leucine.
Phenylalanyl-phenylalanine.
Asparagyl-mono-glycine.
Prolyl-alanine.
α-Aminononoyl-glycine.
α-Aminononoyl-alanine.
α-Aminononoyl-valine.
α-Aminononoyl-leucine.
α-Aminononoyl-asparagine.
α-Aminolauryl-glycine.
α-Aminolauryl-alanine.
α-Aminolauryl-valine.
α-Aminolauryl-leucine.
α-Aminolauryl-asparagine.

Optically Active.

Glycyl-d-alanine.
Glycyl-d-aminobutyric acid.
Glycyl-l-aminobutyric acid.
Glycyl-d-valine.
Glycyl-l-leucine.
Glycyl-d-isoleucine.
Glycyl-l-isoleucine.
Glycyl-l-phenylalanine.
Glycyl-l-tyrosine.
Glycyl-3, 5-diiodo-l-tyrosine.
Monoglycyl-l-cystine.
Glycyl-l-aspartic acid.
Glycyl-d-glutamic acid.
Glycyl-d-tryptophane.
l-Alanyl-glycine.
dl-Alanyl-d-alanine.
d-Alanyl-d-alanine.
d-Alanyl-d-valine.
d-Alanyl-l-leucine.
d-Alanyl-d-isoleucine.
d-Alanyl-l-tyrosine.
d-Alanyl-3, 5-diiodo-l-tyrosine.
d-Alanyl-d-tryptophane.
d-Aminobutyryl-glycine.
d-Aminobutyryl-d-alanine.
l-Valyl-d-valine.
d-Valyl-glycine.
l-Leucyl-glycine.
l-Leucyl-d-alanine.
l-Leucyl-d-valine.
l-Leucyl-l-leucine.
d-Leucyl-l-leucine.
d-Leucyl-d-leucine.
l-Leucyl-d-leucine.
l-Leucyl-d-isoleucine.
l-Leucyl-l-isoleucine.
mono-l-Leucyl-l-cystine.
l-Leucyl-l-tyrosine.
d-Leucyl-l-asparagine.
l-Leucyl-l-asparagine.
l-Leucyl-l-aspartic acid.
l-Leucyl-d-glutamic acid.
l-Leucyl-l-histidine.
l-Leucyl-l-tryptophane.
d-Leucyl-l-tryptophane.
d-Isoleucyl-glycine.
l-Phenylalanyl-glycine.

Tripeptides.

Diglycyl-glycine (chloracetylchloride + glycyl-glycine ester or + glycine anhydride + NaOH).
Diglycyl-cystine (chloracetylchloride + cystine).
Diglycyl-phenylalanine (chloracetylchloride + glycyl-phenylalanine).
Diglycyl-p-iodophenylalanine (chloracetylchloride + glycyl-p-iodophenylalanine).
Glycyl-leucyl-alanine (chloracetylchloride + leucyl-alanine).
Glycyl-d-alanyl-l-tyrosine (chloracetylchloride + d-alanyl-l-tyrosine).
Glycyl-d-aminobutyryl-d-alanine (chloracetylchloride + d-aminobutyryl-d-alanine).
Alanyl-glycyl-glycine (α-bromopropionylbromide + glycyl-glycine ester).
Dialanyl-cystine (α-bromopropionylbromide + cystine).
Alanyl-leucyl-glycine (α-bromopropionylbromide + leucyl-glycine).
d-Alanyl-d-aminobutyryl-glycine (d-bromopropionylchloride + d-aminobutyryl-glycine).
Dialanyl-alanine (α-bromopropionylbromide + alanyl-alanine).
l-Alanyl-glycyl-glycine (l-bromopropionylchloride + glycyl-glycine ester).
l-Alanyl-glycyl-glycine (l-bromopropionylchloride + glycyl-glycine).
d-Alanyl-glycyl-l-tyrosine (d-α-bromopropionylchloride + glycyl-l-tyrosine).
d-Alanyl-l-leucyl-d-isoleucine (d-bromopropionylchloride + l-leucyl-d-isoleucine).
d-Aminobutyryl-glycyl-d-aminobutyric acid (d-bromobutyrylchloride + glycyl-d-aminobutyric acid).
d-Aminobutyryl-glycyl-d-alanine (d-bromobutyrylchloride + glycyl-d-alanine).
Leucyl-glycyl-glycine (α-bromisocapronylchloride + glycyl-glycine ester or + glycine anhydride + NaOH).
Leucyl-α-leucyl-phenylalanine (α-bromisocapronylchloride + α-leucyl-phenylalanine).
Leucyl-glycyl-phenylalanine (α-bromisocapronylchloride + glycyl-phenylalanine).
Dileucyl-cystine (α-bromisocapronylchloride + cystine).
Di-l-leucyl-cystine (d-α-bromisocapronylchloride + cystine).
Leucyl-alanyl-glycine A, Leucyl-alanyl-glycine B } (α-bromisocapronylchloride + alanyl-glycine).
Leucyl-alanyl-alanine A, Leucyl-alanyl-alanine B } (α-bromisocapronylchloride + alanyl-alanine).
l-Leucyl-glycyl-l-aspartic acid (d-bromisocapronylchloride + glycyl-l-aspartic acid).
l-Leucyl-glycyl-d-tryptophane (d-α-bromisocapronylchloride + glycyl-d-tryptophane).
l-Leucyl-glycyl-l-leucine (d-α-bromisocapronylchloride + glycyl-l-leucine).
l-Leucyl-l-tryptophyl-d-glutamic acid (d-α-bromisocapronylchloride + l-tryptophyl-d-glutamic acid).
l-Leucyl-glycyl-d-isoleucine (d-α-bromisocapronylchloride + glycyl-d-isoleucine).
Phenylalanyl-glycyl-glycine (phenyl-α-bromopropionylchloride + glycyl-glycine).
Asparagyl-dialanine (fumarylchloride + alanine ester).

Tetrapeptides.

Triglycyl-glycine (chloracetylchloride + diglycyl-glycine).
d-Alanyl-diglycyl-glycine (d-α-bromopropionylchloride + diglyclyglycine).
Dileucyl-glycyl-glycine (α-bromisocapronylchloride + leucyl-glycyl-glycine).
l-Leucyl-diglycyl-glycine (d-α-bromisocapronylchloride + diglycyl-glycine).
Glycyl-d-alanyl-l-leucyl-d-isoleucine (chloracetylchloride + d-alanyl-l-leucyl-d-isoleucine).
Glycyl-l-leucyl-glycyl-l-leucine (chloracetylchloride + l-leucyl-glycyl-l-leucine).

Pentapeptides.

Tetraglycyl-glycine (chloracetylchloride + triglycyl-glycine).
l-Leucyl-glycyl-l-leucyl-glycyl-l-leucine (d-bromisocapronylchloride + glycyl-l-leucyl-glycyl l-leucine).

Method III. By Means of the Acid Chlorides of the Amino Acids and of the Polypeptides.

This, the simplest method of combining together two or more amino acids, is the one, in contradistinction to the previous one, by which the chain of amino acids can be lengthened at the carboxyl end of the molecule. It could not be employed at the commencement of Emil Fischer's researches, since the acid chlorides of the amino acids were unknown, and all attempts to prepare them had failed; but it is now of the greatest importance, as it admits of the preparation of any conceivable polypeptide, and it has also given us the knowledge of the most complex compound known by synthesis.

Although the acid chlorides of the amino acids themselves were unknown, it was found by Fischer that their carbethoxyl derivatives, as also those of the dipeptides which had been prepared, could be converted into their acid chlorides by the action of thionyl chloride, and that these compounds could be combined with the esters of the amino acids or of polypeptides, thus:—

Carbethoxyl-glycyl chloride and glycine ester yielded carbethoxyl-glycyl-glycine ester.
Carbethoxyl-glycyl chloride and glycyl-glycine ester yielded carbethoxyl-diglycyl-glycine ester.
Carbethoxyl-glycyl-glycyl chloride and glycyl-glycine ester yielded carbethoxyl-triglycyl-glycine ester.

This last compound on hydrolysis gave the free acid, which contains four glycyl groups, and was the first known representative of the tetrapeptides.

In the same way derivatives of mixed polypeptides could be obtained, *e.g.*, carbethoxyl-glycyl-alanine ester. From it, by the action of ammonia, Fischer and Otto prepared the amide and, by saponification with soda, the free acid, but the preparation of the simple polypeptide could not be effected, since it was impossible to remove the carbethoxyl group without complete destruction of the molecule.

In 1904 Fischer found that the presence of a halogen acyl group in the molecule of an amino acid again allowed of the preparation of the acid chloride, *i.e.*, when the amino group of the amino acid was rendered stable, and that this compound was formed by the action of phosphorus pentachloride in the presence of acetyl chloride. As before, this acid chloride could be combined with the esters of amino acids or of polypeptides, *e.g.*—

Bromisocapronylglycine was converted into its acid chloride and

combined with glycine ester, when it yielded bromisocapronyl-glycyl-glycine ester,

$$C_4H_9.CHBr.CO{-}NH.CH_2.COCl + 2NH_2.CH_2.COOC_2H_5 = HCl.NH_2.CH_2.COOC_2H_5 + C_4H_9.CHBr.CO-NH.CH_2.CO-NH.CH_2.COOC_2H_5$$

which, on subsequent saponification and treatment with ammonia, gave the tripeptide leucyl-glycyl-glycine,

$$C_4H_9.CH(NH_2).CO-NH.CH_2.CO-NH.CH_2.COOH.$$

The tetrapeptide leucyl-diglycyl-glycine,

$$C_4H_9.CH(NH_2).CO-NH.CH_2.CO-NH.CH_2.CO-NH.CH_2.COOH,$$

was obtained when bromisocapronyl glycine was combined with glycyl-glycine ester and treated in the same way.

Not only was it possible to prepare the acid chloride of a halogen acyl derivative of an amino acid, but also that of a di-, tri-, etc., peptide by exactly the same means. Thus, the compound bromisocapronyl diglycyl-glycyl chloride can be obtained, and by condensing it with the esters of amino acids and of polypeptides Fischer has prepared a hexa-, a hepta-, and a deca-peptide (see tabulation).

These compounds already exhibit the extraordinary possibilities of synthesis by this method. By continuing the process of preparing the acid chloride of a new polypeptide and again combining it with a polypeptide ester, the synthesis of the complex octadecapeptide, composed of fifteen glycine residues and three leucine residues, was effected in 1907. Its preparation is the best illustration of how this method lends itself to the synthesis of the polypeptides.

Bromisocapronyl diglycyl-glycine was converted into its acid chloride and combined with pentaglycyl-glycine. The resulting bromo compound was treated with liquid ammonia and the decapeptide l-leucyl-octaglycyl-glycine,

$$C_4H_9.CH(NH_2).CO-(NH.CH_2.CO)_8-NH.CH_2.COOH,$$

was obtained. This gave the tetradecapeptide, leucyl-triglycyl-leucyl-octaglycyl-glycine,

$$C_4H_9.CH(NH_2)CO-(NH.CH_2.CO)_3-C_4H_9.CH(NH_2).CO-(NH.CH_2.CO)_8-NH.CH_2.COOH,$$

on combination with bromisocapronyl diglycyl-glycyl chloride and subsequent treatment with ammonia.

A repetition of the process of combining this new compound with bromisocapronyl-diglycyl-glycyl chloride and treating with ammonia yielded the octadecapeptide, leucyl-triglycyl-leucyl-triglycyl-leucyl-octaglycyl-glycine,

$$C_4H_9.CH(NH_2)CO-(NH.CH_2.CO)_3-C_4H_9.CH(NH_2).CO-(NH.CH_2.CO)_3-C_4H_9.CH(NH_2).CO-(NH.CH_2.CO)_8-NH.CH_2.COOH.$$

In the preparation of this octadecapeptide complete combination of the polypeptide with the acid chloride was very essential, since if the greater part of the compound were not used up, but remained unchanged, it was precipitated with the bromo compound on acidifying; this was only attained by using a very large excess of the acid chloride. At the same time there was the technical difficulty of frothing; this was overcome by shaking with glass beads in large flasks. Liquid ammonia was also necessary for the conversion of the halogen compound into its amino derivative. Analysis of the polypeptides hardly sufficed for the determination of their synthesis, since the variations in the figures are so small, but a determination of the bromine in the corresponding halogen derivative indicated that the synthesis was being effected in the stages represented.

This octadecapeptide has the highest molecular weight of any compound as yet prepared by synthesis and of which we know the constitution. Its molecular weight is 1213, a figure which far exceeds that of the fats, tristearin having a molecular weight of only 891. If the compound contained other amino acid residues, such as leucine, tyrosine, phenylalanine in the place of the glycine residues, the molecular weight would be increased two to three times.

Such a figure of 3,000-5,000 has been found for the molecular weight of many proteins, and it would appear that they are composed of some twenty amino acids. The higher values of 12,000-15,000 which have been found for the molecular weight of other proteins, are, according to Fischer, very doubtful, since we have no indication of their purity, in spite of the crystallisability of many of them; the admixture of a small quantity of another protein might easily raise the value to this extent.

Fischer subsequently found that the acid chloride of other acyl derivatives of the amino acids could be prepared by the same process. Thus, by treating finely powdered hippuric acid with phosphorus pentachloride in the presence of acetyl chloride, he obtained hippuryl chloride, a compound which numerous investigators had tried to synthesise, but unsuccessfully. By combining hippuryl chloride with glycine ester, benzoyl-glycyl-glycine was obtained, and this compound, when converted into its acid chloride and combined with glycine, yielded benzoyl-diglycyl-glycine. In this way, by means of hippuric acid chloride, Fischer has prepared the same compounds which Curtius has prepared by means of hippurazide. Max has since prepared the acid chlorides of benzoylalanine, benzoyl-leucine, etc.

By applying this method of preparing the acid chlorides to the amino acids themselves, Fischer ultimately succeeded in obtaining the amino acid chlorides, so that he was enabled to combine together any two amino acids in any order, without the necessity of preparing the corresponding halogen derivative. Polypeptides containing the natural optically active amino acids can thus be synthesised with ease, since the natural compound obtained by hydrolysis can be again used directly in the synthesis, and very often it is easier to prepare the natural compound than the synthetical one, which also requires separation into its stereoisomers.

This method cannot be used directly for the introduction of tyrosine and other oxyamino acids on account of the reactivity of the hydroxyl group with phosphorus pentachloride. If, however, the hydroxyl group be protected by combination with the carbomethoxyl group, the acid chloride can be prepared and used for synthesis. The carbomethoxyl group is subsequently readily removed by saponification. In this way Fischer, in 1908, prepared glycyl-tyrosyl-glycyl-alanine:— chloracetyl-l-tyrosine was shaken in alkaline solution with methyl chlorocarbonate, when chloracetyl-carbomethoxy-l-tyrosine,

$$\begin{array}{l} Cl.CH_2.CO{-}NH.CH.COOH \\ \qquad\qquad\qquad\quad | \\ \qquad\qquad\qquad\quad CH_2.C_6H_4.O.COOCH_3 \end{array}$$

was obtained.

Treatment of this compound with acetyl chloride and phosphorus pentachloride gave the corresponding acid chloride, which on combination with glycine ester yielded chloracetyl-carbomethoxy-tyrosyl-glycine ester,

$$\begin{array}{l} Cl.CH_2.CO{-}NH.CH.CO{-}NH.CH_2.COOC_2H_5 \\ \qquad\qquad\qquad\quad | \\ \qquad\qquad\qquad\quad CH_2.C_6H_4.O.COOCH_3 \end{array}$$

Complete saponification occurred on shaking the ester with dilute alkali; the carbomethoxy group was eliminated as methyl alcohol and carbon dioxide and chloracetyl-tyrosyl-glycine,

$$\begin{array}{l} Cl.CH_2.CO{-}NH.CH.CO{-}NH.CH_2.COOH \\ \qquad\qquad\qquad\quad | \\ \qquad\qquad\qquad\quad CH_2.C_6H_4.OH \end{array}$$

was formed.

Glycyl-tyrosyl-glycine resulted on treatment with ammonia.

The tetrapeptide, glycyl-tyrosyl-glycyl-alanine, was prepared in the same way by employing glycyl-d-alanine ester instead of glycine ester. Racemisation unfortunately occurred in the process: the chloracetyl-carbomethoxy-tyrosyl-glycine was optically inactive and also

the further products: the glycyl-tyrosyl-glycyl-alanine though active is probably a mixture of two stereoisomers.

Formyl tyrosine can also be used in the syntheses by this method after combination with methyl or ethyl chlorocarbonate.

Polypeptides containing tyrosine are of the greatest interest, since the first natural tetrapeptide was isolated from silk in 1907 and was composed of two molecules of glycine, one molecule of alanine and one molecule of tyrosine; and it behaved like the secondary proteoses with ammonium sulphate. Twelve isomers are possible for a tetrapeptide of this composition; this number is reduced to eight if the results of partial hydrolysis and subsequent anhydride formation be taken into account (p. 82). Of these, the synthesis of glycyl-tyrosyl-glycyl-alanine has just been described; glycyl-d-alanyl-glycyl-l-tyrosine was synthesised in 1908 by combining chloracetyl-d-alanyl-glycyl chloride with l-tyrosine ester :—

$$Cl.CH_2.CO—NH.CH(CH_3).CO—NH.CH_2.COCl + 2NH_2.CH.CH_2(C_6H_4OH).COOCH_3 = HCl.NH_2.CH.CH_2(C_6H_4OH).COOCH_3 + Cl.CH_2.CO—NH.CH(CH_3).CO—NH.CH_2.CO—NH.CH.CH_2(C_6H_4OH).COOCH_3$$

saponifying the resulting chloro compound with caustic soda and treating with aqueous ammonia :—

$$Cl.CH_2.CO—NH.CH(CH_3).CO—NH.CH_2.CO—NH.CH.CH_2(C_6H_4OH).COOCH_3 + H_2O + 2NH_3 = CH_2OH + NH_4Cl + NH_2.CH_2.CO—NH.CH(CH_3).CO—NH.CH_2.CO—NH.CH.CH_2(C_6H_4OH).COOH$$

This product, though it had many points of resemblance with the natural tetrapeptide, such as precipitation by phosphotungstic acid, tannic acid, hydrolysis by trypsin, was not identical with it; it differed mainly in its behaviour to ammonium sulphate, by which it was only salted out with great difficulty; the first compound showed closer relationship to the proteoses with ammonium sulphate.

An attempt was made at the same time to prepare the isomeric d-alanyl-glycyl-glycyl-l-tyrosine; it failed on account of the difficulty of preparing pure *a*-bromopropionyl-glycyl-glycyl chloride, but there seems no reason to suppose that Fischer will not overcome this small difficulty in preparing a desired compound, when he has overcome such vast difficulties already in connection with the synthesis of the polypeptides.

This method is most useful in the synthesis of those polypeptides containing amino acids having no corresponding halogen derivative of easy access, e.g., tryptophane, proline. Abderhalden has prepared l-tryptophyl-glutamic acid and l-leucyl-l-tryptophyl-glutamic acid.

This tripeptide was not identical with a natural one isolated from the products of the partial hydrolysis of edestin (p. 83).

Fischer and van Slyke have combined α-pyrrole-carboxylic acid, which is closely related to proline, with glycine in this way.

The accompanying table gives a list of the compounds prepared by this method.

POLYPEPTIDES SYNTHESISED BY METHOD III.

Dipeptides.

Benzoylglycyl-glycine (hippuryl chloride + glycine ester).
d-Alanyl-glycine (d-alanyl chloride + glycine ester).
d-Alanyl-d-alanine (d-alanyl chloride + d-alanine ester).
dl-Valyl-glycine.
Valyl-alanine A.
l-Leucyl-glycine (l-leucyl chloride + glycine ester).
l-Leucyl-d-alanine (l-leucyl chloride + d-alanine ester).
l-Leucyl-l-leucine (l-leucyl chloride + l-leucine ester).
l-Prolyl-l-phenylalanine (l-prolyl chloride + l-phenylalanine ester).
l-Prolyl-d-phenylalanine (l-prolyl chloride + d-phenylalanine ester).
α-Pyrroyl-glycine (α-pyrroyl chloride + glycine ester).
Pyrrolidonyl-glycine (pyrrolidonyl chloride + glycine ester).
d-Tryptophyl-glycine (d-tryptophyl chloride + glycine ester).
l-Tryptophyl-d-glutamic acid (l-tryptophyl chloride + d-glutamic acid ester).

Tripeptides.

Benzoyl-diglycyl-glycine (benzoyl-glycyl-glycyl chloride + glycine ester).
Leucyl-glycyl-glycine (leucyl-glycyl chloride + glycine ester).
Leucyl-glycyl-leucine (leucyl-glycyl chloride + leucine ester).
l-Leucyl-l-tryptophyl-d-glutamic acid (l-leucyl-l-tryptophyl chloride + d-glutamic acid ester).
l-Leucyl-glycyl-d-alanine (d-bromisocapronyl-glyclyl chloride + d-alanine ester).
l-Leucyl-glycyl-l-leucine (d-bromisocapronyl-glycyl chloride + l-leucine ester).
Glycyl-l-asparagyl-l-leucine (chloracetyl-l-asparagyl chloride + l-leucine ester).
Glycyl-tyrosyl-glycine (chloracetyl-carbomethoxy-tyrosyl chloride + glycine ester).

Tetrapeptides.

Leucyl-diglycyl-glycine (bromisocapronyl-glycyl-glycyl chloride + glycine ester).
„ „ „ (bromisocapronyl-glycyl chloride + glycyl-glycine ester).
„ „ „ (leucyl-diglycyl chloride + glycine ester).
d-Alanyl-glycyl-l-tyrosine (bromopropionyl-glycyl-glycyl chloride + l-tyrosine ester).
Glycyl-d-alanyl-glycyl-l-tyrosine (chloracetyl-d-alanyl-glycyl chloride + l-tyrosine ester).
Glycyl-tyrosyl-glycyl-alanine (chloracetyl-carbomethoxy-tyrosyl chloride + glycyl-d-alanine ester).
Glycyl-glutamyl-diglycine (chloracetyl-glutamyl dichloride + glycine ester).
Glycyl-asparagyl-diglycine (chloracetyl-asparagyl dichloride + glycine ester).

Pentapeptides.

Leucyl-triglycyl-glycine (α-bromisocapronyl-diglycyl-glycyl chloride + glycine ester).
l-Leucyl-triglycyl-l-leucine (α-bromisocapronyl-diglycyl-glycyl chloride + l-leucine).

Hexapeptide.

Leucyl-tetraglycyl-glycine (α-bromisocapronyl-diglycyl-glycyl chloride + glycyl-glycine).

Heptapeptide.

Leucyl-pentaglycyl-glycine (α-bromisocapronyl-diglycyl-glycyl chloride + diglycyl-glycine).

Octapeptide.

Leucyl-hexaglycyl-glycine (α-bromisocapronyl-diglycyl-glycyl chloride + triglycyl-glycine).

Decapeptide.

Leucyl-octaglycyl-glycine (α-bromisocapronyl-diglycyl-glycyl chloride + pentaglycyl-glycine).

Dodecapeptide.

Leucyl-decaglycyl-glycine (bromisocapronyl-tetraglycyl-glycyl chloride + pentaglycyl-glycine).

Tetradecapeptide.

Leucyl-triglycyl-leucyl-octaglycyl-glycine (bromisocapronyl-diglycyl-glycyl chloride + l-leucyl-octaglycyl-glycine).

Octadecapeptide.

Leucyl-triglycyl-leucyl-triglycyl-leucyl-octaglycyl-glycine (bromisocapronyl-diglycyl-glycyl chloride + leucyl-triglycyl-leucyl-octaglycyl-glycine).

The Structure of the Polypeptides and Diketopiperazines.

From the methods by which the polypeptides are obtained by synthesis it can only be concluded that their constituent amino acids are combined together in the form of acid amides; this method of combination also occurs in the case of the oxyamino acids, *e.g.*, leucyl-isoserine, where the ester method of combination was excluded by special precautions. The question of their structure still remains very complex, if the controversy concerning the structure of the amides and amino acids, which has not yet been settled, be taken into account. There is the possibility of lactam and lactim forms and of the free amino acid and intramolecular salt; these are illustrated by the four formulæ for glycyl-glycine :—

$NH_2.CH_2.CO{-}NH.CH_2.COOH$ $\quad$ $NH_2.CH_2.C(OH){=}N.CH_2.COOH$

$NH_2.CH_2.CO{-}NH.CH_2.COO$ $\quad$ $NH_2.CH_2.C(OH){=}N.CH_2.COO$

For the sake of simplicity and since his observations have as yet led to no choice between the above formulæ, Fischer has adopted the first formula, but in certain of the polypeptides the observations suggest different structures, thus leucyl-diglycyl-glycine in its amorphous state is easily soluble in alcohol; if the alcohol solution be warmed on the water bath, the crystalline tetrapeptide commences to separate out and in this state it is insoluble in alcohol.

Polypeptides which contain amino dicarboxylic acids or diamino acids can also exist in isomeric forms; asparagyl-monoglycine can exist in the two forms,

$CO{-}NH.CH_2.COOH$ $\quad$ and $\quad$ $COOH$

$CH.NH_2$ $\quad$ $CH.NH_2$

$CH_2.COOH$ $\quad$ $CH_2.CO{-}NH.CH_2.COOH$

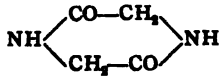
as also the dipeptide of diaminopropionic acid :—

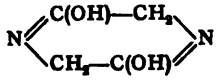
$NH_2.CH_2.CH(NH_2).CO{-}NH.CH_2.CH(NH_2).COOH$

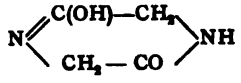
$NH_2.CH_2.CH(NH_2).CO{-}NH.CH.COOH$

$CH_2.NH_2$

The diketopiperazines which are so closely related to the dipeptides can also occur in a keto- or -enol form; the possibilities are :—







The existence of the -enol form was emphasised by the fact that in the hydrolysis of alanine anhydride by alkali, a transient formation of an alkali compound was observed.

The Configuration of the Polypeptides.

Excepting glycine all the amino acids employed in the previous syntheses contain an asymmetric carbon atom. According to the law of Van't Hoff, the polypeptides will occur in 2^n forms. Thus, a dipeptide,

$$NH_2.CHR.CO—NH.CHR.COOH,$$

containing two asymmetric carbon atoms will be capable of existence in the four active forms

dd′ ll′ dl′ ld′

of which the two first and the two last together will form a racemic compound.

A tripeptide can exist in 2^3 or eight forms, a tetrapeptide in 2^4 or sixteen forms, etc.

The two inactive forms of a dipeptide are obtained when the two optically inactive units are coupled together by synthesis, and they appear first in the form of the corresponding halogen derivative,

$$Br.CHR.CO—NH.CHR.COOH.$$

A separation of the two racemic forms has been effected in certain cases at this stage, *e.g.*, leucyl-phenylalanine, but in the majority of cases only one product has been isolated. The formation of only one product in the reaction may be due either to the influence of stereo-isomerism upon the combination of the units, which is especially noticeable when enzymes are concerned, or it may be due to a difference in the rate of combination, an observation first made by Markwald and Mackenzie. The latter explanation is the more probable, since when both compounds have been isolated their amounts have been very different. There remains the possibility that the single substance isolated is still a mixture of the two compounds, as it is so difficult to separate a mixture of substances having similar and almost identical properties.

The two racemic compounds when they have been isolated are distinguished by terming the more insoluble compound A and the more soluble compound B. By the action of trypsin, which only hydrolyses the compound containing the naturally occurring amino acids, it has been possible to determine what the combinations present in these compounds are; thus, as alanyl-leucine A was hydrolysed by trypsin it must contain d-alanyl-l-leucine, and the two racemic compounds will be

d-alanyl-l-leucine
l-alanyl-d-leucine } A

d-alanyl-d-leucine
l-alanyl-l-leucine } B

This has been proved by the later work upon the optically active polypeptides composed of these amino acids.

Only one product can result when the two components consisting of the pure optically active amino acids are combined together, *e.g.*— d-alanyl-d-alanine from d-alanyl chloride and d-alanine ester.

Two products result when one of the components is optically active and the other racemic. The various combinations of optically active tyrosine and aspartic acid with racemic leucine, alanine, etc., come into this category; they are designated as, *e.g.*—

dl-alanyl-l-tyrosine
glycyl-dl-leucine

These compounds are not optical antipodes and can be separated by simple crystallisation. The separation of the leucyl-asparagines,

d-leucyl-l-asparagine
l-leucyl-l-asparagine

has been effected, but in the majority of cases no separation was carried out. The similarity of the isomers is so great that they conform to the condition termed by Fischer in 1894 "partial racemism". It occurs almost always when a racemic compound in combination with an active residue cannot be separated into its two isomeric forms by simple crystallisation.

Cystine is peculiar, and resembles the tartartic acids in its stereochemistry as its constitution shows,

$$COOH.CH(NH_2).CH_2.S.S.CH_2.CH(NH_2).COOH.$$

It is composed of two exactly similar halves, and it matters very little with which amino group combination is effected, but if it be combined with two molecules of a racemic acid chloride, *e.g.*, *a*-bromopropionyl chloride, three isomeric optically active compounds can result, namely:—

d-bromopropionyl-d-bromopropionyl-cystine
l-bromopropionyl-l-bromopropionyl-cystine
d-bromopropionyl-l-bromopropionyl-cystine.

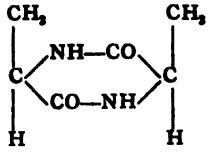
A yield of 71 per cent. of dibromopropionyl-cystine was obtained by Fischer and Suzuki and was apparently a definite substance. It was therefore regarded as the dl-compound, since its formation is independent of the formation of the dd- or ll-compounds which most probably would result in equal amounts.

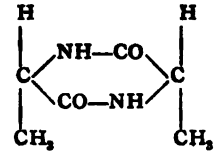
The Configuration of the Anhydrides.

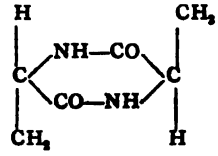
Diketopiperazines composed of two molecules of the same amino acids, *i.e.*, containing the same substituting group, according to theory, can exist in four forms, which are comparable to those of the tartaric acids, namely :—

1. The active dextro form, containing the two dextro rotating molecules.
2. The active laevo form, containing the two laevo rotating molecules.
3. The inactive form, a mixture of 1 and 2.
4. The inactive form, containing a dextro and a laevo rotating molecule.

The following are the structural formulæ for the anhydrides of alanine :—







The substituting groups in the optically active forms are in the *cis*-position, in the inactive meso form in the *trans*-position.

Fischer and Raske in 1906 showed that these various forms of the alanine anhydrides existed. They prepared the inactive *trans*-anhydride,

1. by the action of ammonia upon l-alanyl-d-alanine ester ;
2. by the action of ammonia upon d-alanyl-l-alanine ester.

The active d-alanine anhydride had been previously synthesised in a similar way from d-alanyl-d-alanine ester, and an inactive anhydride had been obtained by heating inactive alanine ester ; the latter probably represents the inactive mixture of the dextro and laevo anhydrides ; the remaining compound, l-alanine anhydride, has not yet been prepared.

The several forms of the diketopiperazines were synthesised in 1907 by Fischer and Koelker, who prepared :—

1. d-leucine anhydride from d-leucyl-d-leucine ester and ammonia ;
2. l-leucine anhydride from l-leucyl-l-leucine ester and ammonia ;

3. *trans*-leucine anhydride from d-leucyl-l-leucine ester and ammonia and from l-leucyl-d-leucine ester and ammonia.

Hydrolysis of these anhydrides by alkali should give the corresponding dipeptide, but in the case of the aminobutyryl-aminobutyric acid anhydrides Fischer and Raske have found that a steric rearrangement occurred; the dipeptide A (p. 47) was obtained both from the anhydride A and B; these had been prepared by the action of ammonia on the respective inactive esters, and in this manner the dipeptide B can be converted into the dipeptide A.

The number of isomers of diketopiperazines containing two molecules of different amino acids is greater than when the two amino acids in the molecule are the same. It can be calculated from the number of asymmetric carbon atoms in the molecule just as in the open chain compounds; thus alanyl-leucine anhydride can exist in four optically active forms and two racemic forms:—

1. d-alanyl-d-leucine anhydride;
2. d-alanyl-l-leucine „
3. l-alanyl-d-leucine „
4. l-alanyl-l-leucine „
5. a mixture of 1 and 4;
6. a mixture of 2 and 3.

The same diketopiperazines are obtainable either from the dipeptides alanyl-leucine or from the dipeptides leucyl-alanine, so that in fact the number of isomers of a diketopiperazine composed of two different amino acids is less than those of the isomers of the dipeptide composed of the same two amino acids.

At present only a racemic form prepared from leucyl-alanine by fusion has been obtained; its nature has not been determined.

The Properties of the Polypeptides.

I. Solubility, Optical Properties, etc.

The physical properties of the various polypeptides show generally much resemblance to one another, although many differences have been observed.

The majority are easily soluble in water; the exceptions amongst the dipeptides are, dl-leucyl-glycine, leucyl-alanine and leucyl-leucine; also phenylalanyl-glycine, phenylalanyl-phenylalanine and some others; amongst the tripeptides, leucyl-alanyl-alanine A, phenyl-alanyl-glycyl-glycine, leucyl-glycyl-phenylalanine; amongst the tetra-peptides, dileucyl-glycyl-glycine. In contradistinction to the other polypeptides made up entirely of glycine units, the pentapeptide and the hexapeptide are soluble with difficulty even in hot water.

Of the complex polypeptides, the octapeptide, l-leucyl-hexaglycyl-glycine, is the most soluble in water, and the decapeptide, l-leucyl-octaglycyl-glycine, the least soluble; the solubility increases again in the case of the tetradeca- and octadecapeptides; their warm clear aqueous solutions become opalescent on cooling.

In general, the solubility in water of polypeptides containing different units is greater than the solubility of the polypeptides made up of a single amino acid; the ready solubility of the dipeptides glycyl-l-tyrosine and leucyl-tyrosine which contains the amino acids soluble with difficulty in water, should be noted.

Most of the polypeptides are insoluble in alcohol. Certain poly-peptides, for instance leucyl-diglycyl-glycine, in the amorphous state are soluble in alcohol, but they are changed on warming into their insoluble crystalline form.

Those polypeptides which are soluble with difficulty in water are dissolved easily by mineral acids and alkalies with the formation of salts; they are less soluble in acetic acid. In many cases they may be dissolved in alcohol if a few drops of ammonia be added; they separate out on boiling off the ammonia.

The high molecular polypeptides, such as the octa-, the deca-, the dodeca- and the tetradeca-peptides give salts with mineral acids which are soluble with difficulty; only the lower ones give soluble salts.

Most of the polypeptides melt above 200° C. and at the same time undergo decomposition. The dipeptides are converted into their diketopiperazines when they are fused. Certain of the glycine poly-peptides are decomposed without melting.

The taste of the polypeptides is not sweet, like that of the amino acids, but slightly bitter; some of the isomeric polypeptides possess distinct differences in their taste; thus leucyl-alanine is tasteless, but the two alanyl-leucines have a bitter taste. The presence of *a*-amino acids amongst polypeptides may even be recognised by their sweet taste, and the resemblance of the polypeptides to the natural peptones in their bitter taste is very remarkable.

The optically active polypeptides have generally a very high specific rotation in comparison with the amino acids; but the rotation is very changeable just as in other classes of compounds. Mutarotation has not been observed. This property has proved very valuable in the study of the hydrolysis of the polypeptides by the action of enzymes.

The chemical properties of the polypeptides depend greatly on their complexity. Like amino acids, all the ordinary polypeptides, when their solutions are boiled with precipitated copper oxide, give blue, sometimes blue-violet solutions, and in this way differ from the diketopiperazines, whose solutions remain colourless, *i.e.*, they do not give copper salts. Kober is now studying these copper salts.

II. Reactions.

The simple polypeptides, like the *a*-amino acids, give no precipitate with phosphotungstic acid, but this condition depends on the length of the polypeptide chain. Many tripeptides, such as leucyl-glycyl-glycine, give a precipitate with phosphotungstic acid in the presence of sulphuric acid if their solution be not too dilute, and this occurs with almost all the tetrapeptides. The derivatives of the diamino acids behave as expected in giving a precipitate with phosphotungstic acid.

The octa-, deca-, etc., peptides are immediately precipitated by phosphotungstic acid; they are also thrown down by tannic acid and by concentrated ammonium sulphate solutions. They resemble, in fact, many natural proteins and would have been regarded as such if they had been found in nature. They lack only the colour reactions due to the absence of tyrosine, tryptophane, etc., in their molecules.

Di-l-leucyl-cystine, leucyl-triglycyl-tyrosine, and other tripeptides containing tyrosine, resemble the proteoses very closely in their behaviour with ammonium sulphate.

The biuret reaction is positive with the greater number of the polypeptides excluding the dipeptides. In the case of the glycine compounds it occurs first with the tetrapeptide, but it occurs with other tripeptides. It is distinctly more intense as the length of the poly-

peptide chain increases, and the colour is also more intense when the carboxyl group is esterified; this is especially noticeable in the case of triglycyl-glycine and its ester, the so-called biuret base. The same occurs when the carboxyl group is converted into the acid amide group; here one of the conditions necessary, as shown by Schiff, is added.

The polypeptides are attacked by nitrous acid with evolution of nitrogen. Fischer found that the imino group as well as the amino group of leucyl-isoserine and glycyl-leucine was attacked by hydrochloric acid and sodium nitrite, but Abderhalden and van Slyke, who have tested a large number of polypeptides by van Slyke's method (Part I., p. 69) have found that the amino group only is attacked. The following are some of the data they obtained :—

	Amino N.		Total N.
	Found.	Calculated.	Calculated.
Dipeptides :—			
Glycyl-asparagine	9·12	7·41	22·23
Glycyl-phenylalanine	7·74	6·34	12·68
Glycyl-tryptophane	5·96	5·36	16·07
Alanyl-glycine	9·83	9·60	19·20
Alanyl-glycine (from silk)	9·46	9·60	19·20
Alanyl-alanine	8·49	8·75	17·50
Alanyl-leucine	7·32	6·94	13·88
Valyl-glycine	7·76	8·05	16·09
Valyl-alanine	7·38	7·45	14·90
Leucyl-isoserine	6·76	6·43	12·86
Leucyl-tyrosine anhydride	0·00	0·00	9·52
Phenylalanyl-glycine	6·54	6·34	12·68
Tripeptides :—			
Diglycylglycine	9·56	7·41	22·24
Alanyl-glycyl-glycine	6·53	6·90	20·71
Alanyl-leucyl-glycine	5·66	5·41	16·21
Leucyl-glycyl-glycine	5·68	5·71	17·14
Leucyl-tryptophyl-glutamic acid (from edestin)	3·25	3·14	12·56
Tetrapeptides :—			
Triglycyl-glycine	6·79	5·69	22·77
Leucyl-diglycyl-glycine	4·74	4·64	18·56
Higher Polypeptides :—			
Leucyl-octaglycyl-glycine	1·96	2·17	21·75
Leucyl-triglycyl-leucyl-octaglycyl-glycine	1·47	1·51	21·13

These values all agree closely with the theoretical values except in the cases where glycine contains the amino group and stands at the commencement of the chain. If the values obtained be multiplied by the factor 0·8 the true figure results. A differentiation of the amino and imino groups in the polypeptide molecule is thus possible with nitrous acid.

The polypeptides are not acted upon by potassium permanganate

in the cold, but on boiling they are oxidised, as was shown by Pollak in the case of glycyl-glycine.

The synthetical polypeptides are completely hydrolysed by boiling with concentrated hydrochloric acid for five hours; 10 per cent. hydrochloric acid at 100° C. hydrolyses them very slowly, and normal alkali has only a very slight action. Their hydrolysis by enzymes, especially by trypsin, is of such importance that a special section is required for the description of these results (p. 63).

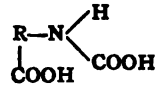
III. Derivatives.

The polypeptides yield the same derivatives as the amino acids. Of the greatest importance are the esters of the polypeptides; they are prepared by the action of alcoholic hydrochloric acid. Hydrolysis of the polypeptide does not occur if prolonged heating be avoided, nor does hydrolysis occur when the esters are saponified by dilute cold caustic alkali. The esters have served in particular for the further synthesis of polypeptides and for the isolation of dipeptides from mixtures; on treatment with alcoholic ammonia, the dipeptide esters are converted into their diketopiperazines. They are not soluble in petroleum ether and they are soluble with difficulty in ether, and they thus differ from amino acid esters. Chloroform dissolves them, and in this solvent their combination with acid chlorides has generally been effected.

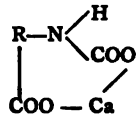
The carboxyl group can be converted into the acid chloride and a halogen acyl group can be attached to the amino group. Further, the benzoyl, the carbethoxyl and the napththalene-sulphonyl compounds can be easily obtained; the derivatives with the last mentioned are soluble with difficulty and may be made use of for characterising the polypeptides. The combination with phenylisocyanate also takes place readily, but these compounds are not so useful as those of the amino acids for purposes of characterisation.

Carbonic Acid Derivatives of Amino Acids and Polypeptides.

The sodium, calcium and barium salts of the monoamino acids have a strongly alkaline reaction and are highly dissociated in solution. If carbonic acid be passed into the solution of the barium salt, barium carbonate is not, as would be expected, immediately formed; the solution remains clear, and only after a short time, when the solution becomes saturated with carbonic acid, does it become cloudy due to the gradual separation of barium carbonate; the precipitation of barium carbonate is hastened by heating. This phenomenon is due, as was shown by Siegfried in 1905, to the formation of salts of carbamino acids of the general formula,



i.e., to the formation of a dibasic acid of which the calcium salt,



is soluble with difficulty in ice-cold water and alcohol. Similar compounds are formed with the dibasic aspartic and glutamic acids and with the diamino acids. In aqueous solutions the free carbamino acid is formed.

The combination of amino acids and other nitrogenous compounds with carbon dioxide is readily ascertained, as was first shown by Siegfried and Neumann in 1908. Carbon dioxide is passed into the solution of the amino compound in lime water, the solution is filtered and boiled. Calcium carbonate separates out. By weighing the calcium carbonate and estimating the nitrogen in the solution the ratio of the two can be determined. If these figures be divided by the molecular weight of calcium carbonate and the atomic weight of nitrogen, the resulting figure gives the number of molecules of carbon dioxide in combination with the number of nitrogen atoms; by reducing the carbon dioxide figure to unity the number of reacting nitrogen atoms is obtained from the quotient

$$\frac{CO_2}{N} = \frac{1}{x}$$

where x is the number of nitrogen atoms taking up one molecule of carbon dioxide.

This quotient in the case of the monoamino acids was 1; in the case of arginine it was $\frac{1}{2}$; in that of histidine $\frac{1}{3}$.

There is thus a distinct regularity in the fixation of carbon dioxide; the amino groups of aliphatic amino acids are quantitatively converted into carbamino groups: in histidine, arginine, tryptophane only the amino group of the side chain, not the nitrogen atoms of the rings, reacts forming a carbamino group.

Amino groups in benzene nuclei do not react except under certain conditions: p-aminophenol reacts quantitatively; p-phenylene diamine does not react quantitatively (Sulze).

Primary amines react quantitatively: tertiary amines do not react.

Not only do amino compounds react, but also alcohols, carbohydrates and hydroxy acids. Carbon disulphide forms similar compounds with amino acids.

Glycyl-glycine was found by Siegfried to react with carbonic acid in the presence of barium hydrate with the formation of the barium salt of glycyl-glycine carbamino acid, which on heating was converted into barium carbonate and glycyl-glycine. Siegfried and Liebermann have found that the peptide linking in the polypeptides reacts to a certain extent. Dipeptides give a quotient of $1 \cdot \frac{1}{63}$ to $1 \cdot \frac{1}{79}$, tripeptides of $\frac{1}{3} \cdot \frac{1}{17}$ and tetrapeptides of $\frac{1}{3} \cdot \frac{1}{29}$, instead of 1, $\frac{1}{3}$ and $\frac{1}{4}$.

Peptones and the proteins of serum as well as the amino acids react with carbonic acid in the presence of calcium salts; this may explain certain of the phenomena concerning the presence of carbonic acid in blood and in working muscle; a protein carbonic acid compound may be formed which can give rise to carbonic acid without taking up oxygen.

The reaction may be made use of for separating glycine and alanine; the calcium salt of glycine carbamino acid is almost insoluble, but the alanine carbamino salt is comparatively soluble. It has been found of use by Siegfried for proving that his intermediate compounds—the kyrines—in the hydrolysis of proteins are not mixtures of amino acids, and for showing that certain peptones can be isolated from solution and their physical properties examined, and proving whether they are still so complex a mixture.

Siegfried's results with glycine and glycyl-glycine have been confirmed by Leuchs, who, in addition, has investigated the combinations of amino acids and of polypeptides with carbonic acid which were prepared by Fischer and his pupils by combination with chlorocarbonic ester.

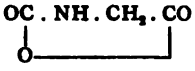
Carbethoxylglycine, which was obtained by combining together chlorocarbonic ester with glycine,

$$Cl.COOC_2H_5 + H_2N.CH_2.COOH = HCl + C_2H_5OOC.NH.CH_2.COOH,$$

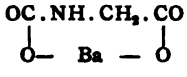
even by careful hydrolysis could not be converted into the free acid, decomposition always occurring with the formation of glycine and carbonic acid; Leuchs, in 1907, obtained the free acid indirectly in the following manner: carbethoxylglycine was converted into its acid chloride,

$$C_2H_5OOC.NH.CH_2.COCl,$$

by the action of thionylchloride, and this compound, when heated, lost ethyl chloride and was changed into the anhydride,



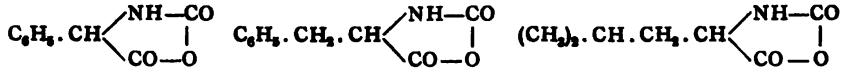
which, when warmed with water to 15° C., decomposed into glycine and carbonic acid, but, when treated with the calculated quantity of baryta, yielded the barium salt of glycine carboxylic acid,



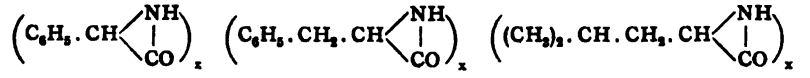
This was identical with the barium salt obtained by Siegfried from glycine, carbonic acid and barium hydrate.

It is of interest to observe that Leuchs found that the anhydride, when treated with a small quantity of water, gave an anhydride of glycine which was not identical with diketopiperazine, but was possibly the same substance which Balbiano and Trasciatti (p. 18) obtained by heating glycine with glycerol, or as that obtained by Curtius from the biuret base (p. 18).

In the same way Leuchs and Geiger, in 1908, obtained the anhydrides of C-phenyl-aminoacetic acid, of phenylalanine and of leucine



by heating the acid chlorides of their carbomethoxyl derivatives, prepared by the action of thionyl chloride, methyl chloride being eliminated in the reaction. On warming these anhydrides in the presence of traces of water, carbon dioxide was evolved with the formation of the following anhydrides,



Carbethoxyl-glycyl-glycine ester was found by Fischer to yield on hydrolysis with alkali the free glycyl-glycine carboxylic acid,

$$HOOC.NH.CH_2.CO.NH.CH_2.COOH,$$

from which, on esterification, an ester was obtained, which was isomeric with the original carbethoxyl-glycyl-glycine ester. This acid was extremely stable in comparison with the glycyl-glycine-N-carboxylic acid obtained by Siegfried in 1906 and also by Leuchs.

The difference between these compounds was shown by Leuchs and Manasse, in 1907, to be due to the fact that the original ester, which has the lactam formula and belongs to the *a*-series,

$$C_2H_5OOC.NH.CH_2.CO.NH.CH_2.COOC_2H_5,$$

undergoes a transformation during hydrolysis and is converted into the acid having the lactim formula and belonging to the *β*-series,

$$C_2H_5OOC.NH.CH_2.C(OH)=N.CH_2.COOC_2H_5.$$

It was proved by the study of the phenyl derivatives,

Carbethoxyl-glycyl-N-phenylglycine ester

$$C_2H_5OOC.NH.CH_2.CO.N(C_6H_5).CH_2.COOC_2H_5,$$

and carbethoxyl-N-phenylglycyl-glycine ester

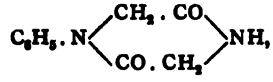
$$C_2H_5OOC.N(C_6H_5).CH_2.CO.NH.CH_2.COOC_2H_5.$$

The former was prepared from carbethoxyl-glycyl chloride and phenylglycine ester, the latter from carbethoxyl-N-phenylglycyl chloride and glycine ester.

Owing to the substitution of the hydrogen atom by phenyl in the position represented in carbethoxyl-glycyl-N-phenylglycine ester, no transformation into the lactim form can take place. On hydrolysis it yielded the dipeptide glycyl-phenylglycine, with loss of carbonic acid,

$$NH_2.CH_2.CO.N(C_6H_5).CH_2.COOH,$$

and this dipeptide was converted on heating into the diketopiperazine,

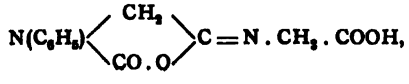


which was also obtained from chloracetylphenylglycine and ammonia.

Carbethoxyl-N-phenyl-glycyl-glycine ester on hydrolysis yields the acid,

$$HOOC.N(C_6H_5).CH_2.C(OH)=N.CH_2.COOH,$$

which does not lose carbon dioxide, and is analogous to Fischer's glycyl-glycine carboxylic acid. Phenyl-glycyl-glycine carboxylic acid easily forms the lactone,



as do also its derivatives; thus carbethoxyl-N-phenyl-glycyl-glycine ester when treated with ammonia yields the amide,

$$C_2H_5OOC.N(C_6H_5).CH_2.C(OH)=N.CH_2.CO.NH_2,$$

which loses alcohol at 220° and forms the lactone,

$$N(C_6H_5)\begin{<}CH_2\\CO.O\end{>}C=N.CH_2.CO.NH_2.$$

The stability of glycyl-glycine carboxylic acid obtained from carbethoxyl-glycyl-glycine ester is therefore due to its having the lactim formula,

$$HOOC.NH.CH_2.C(OH)=N.CH_2.COOH,$$

whereas the instability of glycyl-glycine carboxylic acid obtained from glycyl-glycine and carbonic acid is due to the lactam formula,

$$HOOC.NH.CH_2.CO.NH.CH_2.COOH.$$

Further proof for these formulæ is given by Siegfried's experiments in which he showed that the peptide linking in polypeptides, which have the lactam formula, could also combine with carbonic acid, although glycyl-glycine carboxylic acid which has the lactim formula did not combine with carbonic acid.

Leuchs and La Forge have since shown that the same isomerism is the cause of the existence of two isomeric carboxylic derivatives of diglycyl-glycine esters, namely, the α-form,

$$C_2H_5OOC.NH.CH_2.CO.NH.CH_2.CO.NH.CH_2.COOC_2H_5$$

and the β-form,

$$C_2H_5OOC.NH.CH_2.C(OH)=N.CH_2.C(OH)=N.CH_2.COOC_2H_5.$$

Amides and Imides of Amino Acids and Polypeptides.

The occurrence of asparagine and glutamine in plants and the formation of ammonia by the hydrolysis of proteins points to the presence of amide groups in the protein molecule. This combination may be only with these aminodicarboxylic acids, but there is the possibility that the amide groups may be attached to other units.

Amides of amino acids have therefore been prepared. The earlier investigators only obtained the hydrochlorides of these compounds. Fischer and Koenigs prepared the diamide of aspartic acid. Koenigs and Mylo prepared the amides of glycine, alanine, aminobutyric acid, valine, leucine, phenylalanine and tyrosine by the action of liquid ammonia upon the esters. The reaction took place readily except with valine; in the preparation of glycine amide glycyl-glycine amide was also formed but it was not isolated in a pure state.

Bergell and Wülfing in 1910 found that these amides could be readily obtained by the action of aqueous ammonia upon the esters and that glycine amide was best prepared from chloracetyl amide. Glycine amide hydrochloride was formed by the action of ammonia and the compound itself was obtained by treatment with the calculated quantity of sodium hydrate. The corresponding derivatives—hippuramide and chloracetyl-glycine amide—were obtained when these amides were treated with benzoyl chloride and chloracetyl chloride.

By treating chloracetyl-glycine amide with ammonia they obtained glycyl-glycine amide, and by similar reactions they prepared glycyl-leucine amide and alanyl-leucine amide. Continuing the process of adding on the halogen derivative Bergell and Brugsch have proceeded as far as obtaining chloracetyl-glycyl-leucine amide and chloracetyl-diglycyl-leucine amide. In all these compounds the leucine radicle is at the end of the chain.

Leucine amide was of particular interest: as it gave with copper sulphate and caustic soda a red crystalline compound and it was hydrolysed by trypsin asymmetrically.

The other amides were not hydrolysed by trypsin; leucine amide and alanine amide were hydrolysed by extracts of the liver, spleen and kidney; it seemed that the hydrolysis of leucine amide was effected by a special enzyme which was readily decomposed by acid.

Diglycinimide, $NH_2 . CH_2 . CO—NH—OC . CH_2 . NH_2$, was pre-

pared in 1907 by Bergell. Starting from chloracetamide he obtained chloracetonitrile by heating it with phosphoric anhydride :—

$$Cl.CH_2.CO.NH_2 = H_2O + Cl.CH_2.CN.$$

Dichlorodiacetamide resulted in the interaction of this compound with chloracetic acid,

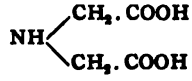
$$Cl.CH_2.CN + HOOC.CH_2Cl = Cl.CH_2.CO-NH-OC.CH_2Cl,$$

and on treating it with ammonia, diglycinimide hydrochloride was obtained,

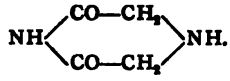
$$Cl.CH_2.CO-NH-OC.CH_2Cl + 3NH_3 =$$
$$NH_4Cl + NH_2.CH_2.CO-NH-OC.CH_2.NH_2.HCl,$$

from which the free base was prepared by means of silver oxide as a crystalline substance of a basic character.

Bergell continued his work on this substance in conjunction with Feigl. Diglycinimide was stable to acids and to the weak alkalies, magnesia and sodium bicarbonate, but it was converted by caustic alkalies and by baryta into ammonia and an acid of the constitution



which was identical with imino diacetic acid, prepared in 1862 by Heintz. Its formation probably took place through the intermediate ring compound,



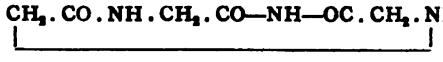
Diglycinimide did not give rise to ammonia and glycine as was expected; but its benzoyl derivative was converted on hydrolysis into hippuric acid, ammonia and glycine :—

$$C_6H_5.CO.NH.CH_2.CO-NH-OC.CH_2.NH_2 + 2H_2O =$$
$$C_6H_5.CO.NH.CH_2.COOH + NH_3 + HOOC.CH_2.NH_2.$$

In order to introduce another glycyl residue into diglycinimide, the chloracetyl derivative was prepared, from which by the action of ammonia glycyl-diglycinimide,

$$NH_2.CH_2.CO.NH.CH_2.CO-NH-OC.CH_2.NH_2,$$

did not result; but ammonia was lost and a compound probably of the formula



was obtained.

The homologous alanyl glycine imide,

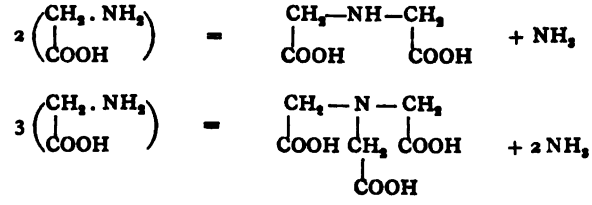
$$CH_3.CH.(NH_2)CO-NH-CO.CH_2.NH_2,$$

was obtained by the action of ammonia at a low temperature upon

methyl dichlorodiacetimide, prepared from chloropropionitrile and chloracetic acid.

Neither diglycinimide nor benzoyldiglycinimide were hydrolysed by pepsin or by trypsin.

Iminodiacetic acid and triglycolamic acid have been shown by Siegfried to be formed in the oxidation of glycine by mercuric chloride at 37° C. :—



The Action of Enzymes.

I. Trypsin.

One of the best proofs that the protein molecule is built up of amino acids coupled together by the methods devised by E. Fischer is given by the action of the various enzymes upon the synthetical polypeptides.

In 1903, soon after the synthesis of a few of the simple dipeptides and their derivatives had been effected, Fischer and Bergell investigated the action of an extract of pancreas upon them, and they found that

glycyl-glycine β-naphthalenesulphonylglycyl-d-alanine β-naphthalenesulphonyl-d-alanyl-glycine di-β-naphthalenesulphonyltyrosyl-dl-leucine	were not hydrolysed
β-naphthalenesulphonylglycyl-l-tyrosine β-naphthalenesulphonylglycyl-dl-leucine carbethoxyl-glycyl-dl-leucine glycyl-l-tyrosine leucyl-alanine alanyl-leucine leucyl-leucine	were hydrolysed

from which it was obvious that several factors conditioned the hydrolysis by the enzymes of the pancreas, such as the nature of the dipeptide and its configuration: *e.g.*, the racemic compounds were hydrolysed asymmetrically, the natural component, such as l-leucine being split off from carbethoxyl-glycyl-dl-leucine, the remainder not being acted upon. The results coincide with the facts known with regard to the rapid separation of leucine and tyrosine from proteins by the action of trypsin; the other amino acids, such as glycine and alanine, are not obtained during the early stages of digestion.

Fischer and Abderhalden, in 1905, extended these observations by investigating the effect of pancreatic juice prepared by Pawlow from a pancreatic fistula and activated by enterokinase from duodenal juice, *i.e.*, by the action of pure trypsin upon a larger number of polypeptides, and they were able to divide the polypeptides into two distinct classes :—

Those Hydrolysed.	Those not Hydrolysed.
* Alanyl-glycine.	Glycyl-alanine.
* Alanyl-alanine.	Glycyl-glycine.
* Alanyl-leucine A.	Alanyl-leucine B.
* Leucyl-isoserine A.	Leucyl-alanine.
Glycyl-l-tyrosine.	Leucyl-glycine.

* These are racemic compounds.

Those Hydrolysed.	Those not Hydrolysed.
Leucyl-l-tyrosine.	Leucyl-leucine.
* Alanyl-glycyl-glycine.	Aminobutyryl-glycine.
* Leucyl-glycyl-glycine.	Aminobutyryl-aminobutyric acid A.
* Glycyl-leucyl-alanine.	Aminobutyryl-aminobutyric acid B.
* Alanyl-leucyl-glycine.	Valyl-glycine.
Dialanyl-cystine.	Glycyl-phenylalanine.
Dileucyl-cystine.	Diglycyl-glycine.
Tetraglycyl-glycine.	Triglycyl-glycine.
Triglycyl-glycine ester (Curtius' biuret base).	Dileucyl-glycyl-glycine.

To which were added in 1907 and later years the following optically active polypeptides:—

d-alanyl-d-alanine.	d-alanyl-l-alanine.
d-alanyl-l-leucine.	l-alanyl-d-alanine.
d-alanyl-l-tyrosine.	l-leucyl-glycine.
l-leucyl-l-leucine.	l-leucyl-d-leucine.
l-leucyl-d-glutamic acid.	d-leucyl-l-leucine.
l-leucyl-l-tyrosine.	
l-prolyl-l-phenylalanine.	
d-alanyl-diglycyl-glycine.	

The hydrolysis of these compounds by the enzyme was determined by the isolation of the individual substances. The isolation of the amino acids soluble with difficulty in water, namely, tyrosine and cystine, presented no great difficulty, since those compounds crystallised out during the process of hydrolysis, but in the other cases the amino acids required separation from unchanged dipeptide. The ester method here again proved its usefulness; the esters of the simple monoamino acids are easily volatile *in vacuo* and can be characterised by the methods previously described; those of the dipeptides are not volatile and are characterised by conversion into their diketopiperazines or anhydrides by the action of ammonia, which compounds are less soluble than the dipeptides themselves and are thus capable of separation by filtration.

By simply determining the change in rotation, especially when optically active polypeptides were investigated, an indication that hydrolysis was occurring was obtained; as soon as the rotatory power became constant it was assumed that complete hydrolysis had taken place and the solution was examined for the products of hydrolysis.

In all cases the activity of the ferment was first proved, and freedom from bacterial infection was specially guarded against and certified by control experiments.

* These are racemic compounds.

An examination of the results of hydrolysis by trypsin shows that several factors have an important influence:—

1. The Structure of the Molecule.

Glycyl-alanine, $NH_2 . CH_2 . CO—NH . CH(CH_3) . COOH$, was not hydrolysed, but the isomeric alanyl-glycine, $NH_2 . CH(CH_3) . CO — NH . CH_2 . COOH$, was hydrolysed; again, alanyl-leucine A was hydrolysed but not leucyl-alanine.

The order in which the amino acids are combined together in the molecule has therefore a very marked effect. Thus, when alanine is the acyl radicle, as in alanyl-glycine, alanyl-alanine and alanyl-leucine, hydrolysis occurred, but the reverse happened when leucine, valine or aminobutyric acid were the acyl radicles; in the cases of leucyl-alanine and leucyl-glycine, no hydrolysis took place; here the racemic compound was employed and the resistance might have been due to this factor, but the instance of l-leucyl-glycine appears to confirm the older result.

If tyrosine, isoserine and cystine stood at the end of the chain, trypsin hydrolysed the compound; in the only case examined where tyrosine, combined with β-naphthalene-sulphonic acid, acted as the acyl radicle, there was no hydrolysis.

2. The Configuration of the Molecule.

This is of the greatest importance in the hydrolysis of a polypeptide by trypsin, as will be seen from the list of compounds published in 1907. Only those compounds containing the naturally occurring optically active variety of the amino acid are hydrolysed by trypsin.

The compounds marked with an asterisk are racemic and their hydrolysis was effected asymmetrically, only that portion of the molecule containing the natural stereoisomer being attacked. This explained the difference between alanyl-leucine A and alanyl-leucine B; the former probably consisted of d-alanyl-l-leucine + l-alanyl-d-leucine, the first of which contains the natural stereoisomers upon which the hydrolysis depends; the latter would consist of d-alanyl-d-leucine + l-alanyl-l-leucine. The later experiments of 1907 proved this supposition. It is more noticeable in the case of leucyl-leucine, which must have been l-leucyl-d-leucine + d-leucyl-l-leucine since l-leucyl-l-leucine is hydrolysed.

3. The Number of Amino Acids.

Only the various polypeptides containing several glycine radicles come at present under this heading. Several interesting details are at once apparent. Hydrolysis first took place when five glycine radicles, as in tetraglycyl-glycine, are combined together, although it occurred in

the ester of triglycyl-glycine, or the biuret base of Curtius, which had been previously examined by Schwarzschild. The length of the glycine chain is therefore of importance, but an alteration in the carboxyl group may have an influence; it is worth noting that Warburg observed that leucine ester was hydrolysed by pancreatic juice, but whether this was due to the trypsin or the lipase in the juice was not determined.

The hydrolysis of leucyl-glycyl-glycine but not of the more complex dileucyl-glycyl-glycine was probably due to the configuration of the dileucyl group.

4. The Nature of the Enzyme.

In the earlier experiments by Fischer and Bergell it was found that leucyl-alanine was hydrolysed by an extract of pancreas; it was not, however, hydrolysed by pure pancreatic juice. Such extracts probably contain other enzymes, more especially the autolytic enzyme which produces the hydrolysis; the later work of Abderhalden and his co-workers upon the action of enzymes from various organs also show that polypeptides not hydrolysed by pure trypsin are attacked by these enzymes (see table, p. 68).

II. Pepsin.

Amino acids have been described by various authors as occurring together with the proteoses and peptones in a pepsin digest of proteins. One might have expected that pepsin would act upon certain of the synthetical compounds, especially those most easily hydrolysed by trypsin. Pure pepsin, prepared by Pawlow, had no action upon glycyl-l-tyrosine, leucyl-alanine, leucyl-leucine, dialanyl-cystine, and leucyl-glycine. Fischer concluded that the chain of amino acids was not sufficiently long to allow of attack by pepsin. The amino acids obtained by the digestion of proteins probably arise by the action of other enzymes contained in the enzyme solution employed.

Another explanation of the non-hydrolysis by pepsin may be that it acts upon combinations of amino acids in the form of anhydrides.

III. Other Enzymes.

Not only are the synthetical polypeptides hydrolysed by the enzyme of the pancreas, but they are also hydrolysed by the enzymes occurring in other parts of the animal body.

It was found by Abderhalden and Bergell, in 1903, that glycyl-glycine when subcutaneously introduced into a rabbit was converted into glycine which appeared in the urine, whereas glycine, if administered in a similar way, was completely burnt up and was not excreted. Abderhalden and Rona subsequently showed that glycyl-l-tyrosine was

burnt up in the organism of the dog when injected into the system, and Abderhalden and Samuely observed that this was also the case when cystine, dialanyl-cystine and dileucyl-cystine were subcutaneously introduced. Abderhalden continued these investigations with Teruuchi, with the result that the organism of the dog was found to be able to utilise completely glycyl-glycine, alanyl-alanine and diglycyl-glycine as well as the diketopiperazines, glycine anhydride and alanine anhydride, when they were given by the mouth, the nitrogen contained in these substances being eliminated as urea. To this series of polypeptides capable of being utilised by the dog Abderhalden and Samuely added dl-leucine and racemic leucyl-leucine, and Abderhalden and Babkin added leucyl-glycine. These results differed from those of Wohlgemuth, who found that the rabbit excreted d-leucine when dl-leucine was given, but Abderhalden and Kautzsch have also found that the rabbit excretes d-leucine when somewhat large doses of dl-leucine are administered, although this animal can utilise dl-leucyl-glycine and dl-leucyl-glycyl-glycine. Abderhalden has since found that the rabbit excretes glycine, l-alanine and d-serine when the diketopiperazines of these amino acids are administered, which points to their hydrolysis into the dipeptide before they are split into the amino acids.

The organs of various animals, such as the dog and rabbit, would thus appear to differ in their power of making use of synthetical polypeptides, but the animal organism as a whole is not so selective as the enzyme of the pancreas which hydrolyses the racemic dipeptide asymmetrically; in the body the racemic compound is completely burnt up, since no dipeptide composed of the optically active variety of the amino acid not occurring in a protein could be isolated. Further, the animal organism is able to utilise polypeptides not hydrolysed by pancreatic juice, so that if such polypeptides are present in the protein they can still be utilised by the body although unaffected by trypsin.

Although these polypeptides are utilised by the organism of an animal and the nitrogen contained in them excreted as urea, it does not follow from the results of the experiments that these polypeptides are hydrolysed into their constituent amino acids before they are absorbed, more especially those which are not acted upon by trypsin.

Great interest therefore is attached to the subsequent work of Abderhalden in conjunction with Teruuchi, Hunter and Rona, which was commenced in 1906 upon the action of extracts and of press-juices of various organs, prepared by Buchner's method of grinding up with sand, mixing with "kieselguhr" and pressing out at a pressure of 100-300

... which the cell enzymes are obtained. A large number ... were employed for determining the nature of these ... in the hope of finding differences between them, and dividing the proteolytic enzymes into definite groups, since we regard enzymes as being extremely selective in their nature, and in the hope also of determining in which organ the hydrolysis of any particular polypeptide took place. The results are given in the following tabulation:—

	Glycyl-glycine.	dl-Leucyl-glycine.	Dialanyl-cystine.	Glycyl-dl-alanine.	Glycine anhydride.	Glycyl-l-tyrosine.	l-Leucyl-d-leucine.	Leucyl-phenylalanine.	dl-Alanyl-glycyl-glycine.	dl-Leucyl-glycyl-glycine.	dl-Alanyl-glycine.	Glycyl-dl-leucine.	Diglycyl-glycine.	Triglycyl-glycine.
Yeast Juice (Endotryptase)	...	...	...	...	...	+	...	...	...	...	...	...	...	...
Juice of Germinating Wheat	+	+	+	...	...	+	...	...	...	...	...	...	...	...
Juice of Germinating Lupine	+	+	+	...	...	+	...	...	...	...	...	...	...	...
Juice of Mushroom	...	+	...	...	...	o	...	...	...	...	+	...	+	...
,, ,, Allescheria Gayonii	...	+	...	...	...	o	+	...	...	...	...	...	...	...
,, ,, of Rhizopus tonkinensis	...	+	...	...	...	...	+	...	...	...	...	...	...	...
,, ,, Aspergillus wentii	...	...	...	...	...	...	+	...	...	...	...	...	...	...
,, ,, Mucor mucedo	...	+	...	...	...	...	o	...	...	...	...	...	...	...
Ox, Liver Extract	+	+	...	...	...	...	...	...	...	...	...	...	...	...
,, ,, Juice	...	+	...	+	o	...	o	+	+	+	...	...	...	...
,, Muscle Juice	+ slight	+ slight	...	+ slight	...	...	...	...	...	...	...	...	...	...
Rabbit, Liver Juice	+	+	...	+	...	...	...	...	...	...	...	...	...	...
,, Kidney Juice	+	+	...	+	...	...	...	...	...	...	...	...	...	...
,, Muscle Juice	+	+	...	+	...	...	...	...	...	...	...	...	...	...
Dog, Muscle Juice	+	...	...	...	...	+	...	...	...	...	...	...	...	...
,, Kidney Juice	+	...	...	...	...	...	...	...	...	...	...	...	...	...
,, Liver Juice	+	...	...	...	...	+	...	...	...	...	...	...	...	...
,, Intestinal Juice (Erepsin)	+	...	...	...	...	+	...	...	...	...	...	...	...	...
,, Extract of Intestine	+	...	...	...	...	...	...	...	...	...	...	...	...	...
Pig, Lenses from Eyes	...	...	...	o	...	+	...	...	...	...	+	...	+	...
Calf, Brain	...	...	...	o	...	o	...	...	...	...	+	...	+	...
Ox, Blood Serum	...	...	...	...	...	o	...	...	...	...	...	...	...	...
Rabbit, Blood Serum	...	...	...	...	...	+	...	...	...	...	...	...	...	...
Dog, Blood Serum	...	...	...	...	...	+	...	...	...	...	...	...	...	...
Horse, Mixed Blood Corpuscles	...	...	...	...	...	+	...	...	+	...	+	+	...	...
Horse, Red Corpuscles	...	...	...	...	...	+	...	...	+	...	+	...	+	...
Dog, Sheep, Rabbit } Red Corpuscles	...	...	...	...	...	+	...	...	...	...	...	...	...	...
Horse, Blood Platelets	...	...	...	...	...	+	...	...	...	...	...	...	...	...
,, Leucocytes	...	...	...	...	...	o	...	...	...	...	...	...	...	...
,, Plasma	o	...	...	o	o	o	...	...	+	o	+	o	+	+
,, Serum	o	...	...	...	o	...	...	...	+	...	+	o	+	+
Ox, Plasma	...	...	...	+	...	o	...	...	...	...	+	...	+	...
,, Red Blood Corpuscles	...	...	...	+	...	+	...	...	...	...	+	...	+	...
,, Blood Platelets	...	...	...	o or slight	...	o	...	...	...	...	o or slight	...	+	...
Saliva	...	+	...	...	...	+	...	...	...	...	+	...	...	...

The enzymes of the various organs of the animal body have hydrolysed with few exceptions all the polypeptides upon which their effect has been studied, but decided differences are noticeable. These enzymes are not so selective in their action as pure pancreatic juice; it was previously found that leucyl-alanine was hydrolysed by an extract of pancreas, but not by the pure juice; glycyl-glycine is not hydrolysed by trypsin, but it is attacked by an extract of liver. Leucyl-leucine was not hydrolysed by the enzymes of the liver of the ox; in all probability this was due to the insolubility of the dipeptide. Another striking result is that glycyl-l-tyrosine was not hydrolysed by the enzymes of calf's brain, which attacked the other polypeptides upon which it was tested. The only diketopiperazine investigated was glycine anhydride, and this was not converted into glycine. This result would point to the absence of anhydrides in the products absorbed from the intestine. In general, the enzymes of the organs are more powerful than trypsin and less selective in their action.

H. Fischer has described the hydrolysis of d-leucyl-l-tryptophane by liver extract and yeast extract. The result is contrary to what was expected as the compound contains in its molecule the unnatural isomer of leucine. Abderhalden attributes the result to the presence of some racemic compound in the specimen employed. Racemic polypeptides are generally hydrolysed asymmetrically, the polypeptide containing the unnatural isomer remaining unattacked.

The proteoclastic enzymes occurring in the germinating seeds of wheat and lupine appear, according to the results obtained by Abderhalden and Schittenhelm, to have a stronger hydrolytic action than trypsin, since they break up glycyl-glycine and dl-leucyl-glycine, which are unaffected by the enzyme of the pancreas. The enzymes in the mushroom and various moulds, although they do not hydrolyse glycyl-l-tyrosine, are able to hydrolyse polypeptides which are not attacked by trypsin. They were tested with the racemic polypeptides and they apparently attack both isomers; glycyl-l-tyrosine seems to be destroyed by other enzymes in the mushroom (Abderhalden and Rilliet, Abderhalden and Pringsheim). The Allescheria mould will grow on media containing various polypeptides as its source of nitrogen.

The most interesting and astonishing facts were obtained by the examination of the blood corpuscles, the plasma and serum. Red blood corpuscles and platelets of the horse (but not of the ox) hydrolysed glycyl-l-tyrosine, which was not attacked by white corpuscles obtained from lymph or from pus cells, nor by the plasma or serum. Plasma

and serum both hydrolysed dl-alanyl-glycine, as also the tri- and tetra-peptides diglycyl-glycine and triglycyl-glycine, which proves that the enzymes in the plasma and serum are not trypsin (or erepsin) absorbed from the intestine. Red blood corpuscles hydrolysed diglycyl-glycine, and the splitting caused by the plasma and serum may be due to the presence of the enzymes of the red blood corpuscles, either excreted naturally, or produced during the separation of the constituents, which was probably not quite perfect, since it is well known that there are great difficulties in obtaining serum or plasma absolutely free from the red colour of the corpuscles.

Applications.

The synthetical polypeptides have been made use of by Abderhalden and his co-workers and other investigators for several purposes:—

I. Detection of Proteoclastic Enzymes.

The hydrolysis of glycyl-l-tyrosine and other dipeptides containing tyrosine or cystine is very readily observed as these compounds contain amino acids, which are soluble with difficulty in water and which gradually crystallise out as hydrolysis proceeds.

Glycyl-l-tyrosine, which can be readily prepared, has therefore been repeatedly used for the purpose of detecting proteoclastic enzymes.

Instead of employing this synthetical dipeptide, Bergell and Schütze suggested the use of the peptone obtained by the hydrolysis of silk. Abderhalden and Schittenhelm [1] have found that it is more convenient than the glycyl-l-tyrosine as it is more soluble in water, contains more tyrosine and is readily prepared. Abderhalden and Steinbeck describe its preparation as follows:—

Silk-cuttings (1 kilo.) are dried at 100° C. for forty-eight hours and then placed in three to five times the quantity of 70 per cent. sulphuric acid. The acid solution is allowed to stand for four days at 25° C., when it is diluted with ten times the quantity of water; the vessel in which it is contained is kept cold with ice during the process of dilution. Sulphuric acid is removed by adding, with continual stirring, the calculated quantity of powdered barium hydroxide; after twelve hours the barium sulphate is filtered off and washed several times with water by stirring up in a mortar and decanting or filtering. The precipitate should not be boiled with water in case neutralisation has not been perfect. The filtrate and washings are tested for barium and sulphuric acid, and when these are found absent, they are evaporated to a small volume *in vacuo* at 40° C.; if frothing occurs the evaporation may be effected by dropping the solution into the evacuated flask through a tap funnel. The solution is now again tested for barium and sulphuric acid. If present they can be removed by warming to 60° C. and adding the exact quantity of the necessary reagent. When these substances are absent, the solution is evaporated *in vacuo* to a thick syrup. The syrup is poured into absolute alcohol when the peptone separates in pale yellow or white flakes. As soon as syrup begins to separate it is

[1] They state that this compound is supplied by Hoffmann La Roche & Co. in Basle under the name "Peptone Roche".

advisable to pour it into a fresh quantity of absolute alcohol. In this way 200-300 grammes of silk peptone can be obtained. More can be obtained by evaporating the alcoholic solution and repeating the precipitation with alcohol.

A purer product may be prepared if the original solution be evaporated *in vacuo* as far as possible, dissolving the residue in hot methyl alcohol and pouring into absolute alcohol. A still purer preparation is obtained by precipitating a one per cent. solution with 10 per cent. phosphotungstic acid solution, decomposing the precipitate in the usual way and evaporating, etc.

The preparation so obtained is easily soluble in water and has generally an amphoteric or slightly acid reaction.

The peptone is used in 10-50 per cent. solution, best 25 per cent. If acid it is carefully treated with sodium bicarbonate till it is just alkaline and filtered, if not clear. The enzyme solution is added and the mixture is kept at 37° C. in presence of toluene. If a proteoclastic enzyme be present tyrosine separates out. Unfortunately the method cannot be used quantitatively as some of the tyrosine is frequently held back in the solution and is not easily separated on concentration.

Numerous organs have been tested with this substrate; proteoclastic enzymes have been detected in the liver, kidney, muscle extracts, stomach contents, fæces. Abderhalden and Heise tested for enzymes in the various groups of invertebrates. All the animals tested contained enzymes which hydrolysed the polypeptide. The parasitic intestinal worms were proved to excrete no enzyme into the lumen of the intestine, though the parasites contained active enzymes in their bodies. Abderhalden and Pringsheim have tested various moulds with the silk peptone. They find with its use that in the method of Buchner for preparing intracellular enzymes there is frequently adsorption of the enzyme by the sand and "kieselguhr".

Abderhalden states that thin slices (one or several) of an organ may be readilytested for proteoclastic enzymes; they are placed in a 25 per cent. solution of the peptone, covered with toluene and kept at 37° C. in a closed vessel. Deposition of tyrosine appears on the surface of the slice and sometimes after twelve hours there may be a considerable amount of deposit. This simple and convenient way of testing for enzymes also serves for their localisation in cells and organs, *e.g.*, medulla and cortex of the kidneys. Using this method Abderhalden has found that proteoclastic enzymes first appeared in seven to eight

days in a developing chick and in pig embryos when they were 3·3 cm. long, but not 3·2 cm. long (about thirty-seven days old). The kidneys and liver contained most of the active enzyme. On account of the wide distribution of the proteoclastic enzymes in tissues no perfect localisation was found possible.

Even simpler than the polypeptides containing tyrosine for detecting enzymes are polypeptides containing tryptophane. These compounds do not react with bromine water, but when hydrolysed the tryptophane is easily recognised by this colour reaction. These polypeptides are not so useful for animal as for vegetable tissues; animal tissues usually give rise to some tryptophane by autolysis and no conclusive result is obtained unless very careful controls are also made.

The use of these polypeptides was suggested by Neubauer and Fischer in 1909 and they used glycyl-l-tryptophane[1] to detect ferments in certain pathological conditions.

Walker Hall and Williamson have found that the gastric contents of a case of cancer of the stomach, the cerebro-spinal fluid in a case of meningitis, and pleuritic exudate hydrolysed glycyl-l-tryptophane. The presence of an enzyme which hydrolyses these polypeptides is, according to Kuttner and Pulvermacher, not a specific test for cancer of the stomach. Koelker mentions that the enzyme probably comes from the saliva; normally it is destroyed by acid in the stomach but owing to the absence of acid in certain cases its activity may be the cause of positive results.

Abderhalden has tested glycyl-l-tryptophane, l-tryptophyl-glycine, d-alanyl-tryptophane, l-leucyl-l-tryptophane, l-leucyl-glycyl-l-tryptophane and l-tryptophyl-glutamic acid with pancreas extract, yeast juice and other enzyme solutions. All were hydrolysed.

Glycyl-l-tryptophane was used by Jacoby to detect enzymes in the fertilised and developing eggs of Arbacia; by Koblanck and Löb in the ovaries of the rabbit and pig.

This test may also be carried out with slices of the organs: they are placed in a 1 per cent. solution of the dipeptide, removed after twelve to forty-eight hours and the solution is tested for tryptophane by the bromine water reaction.

Solutions of the optically active polypeptides can be employed with more certainty. It simply suffices to place the polypeptide and enzyme solutions in a polarimeter tube and observe the change in rota-

[1] Kalle & Co. in Biebrich supply this compound under the term "ferment diagnostikon".

tion. A control should be made of the optical changes occurring in the enzyme solution under the same conditions.

The change in rotation of the glycyl-l-tyrosine or silk peptone solution may be observed as well as the separation of tyrosine. In testing for enzymes in various bacteria Abderhalden, Pincussohn and Walther used not only silk peptone, but peptones prepared in the same way from edestin, gelatin and egg albumin as substrate, observing the change in rotation as the means of detection. Though the results were in general the same certain differences could be observed.

Koelker has used racemic alanyl-glycine as substrate for testing the activity of a yeast extract in an attempt to separate out the proteoclastic enzyme.

Kober, who has found that alkali precipitates copper hydrate from the copper salts of amino acids, suggests that this reaction might be used for detecting proteoclastic enzymes, as dipeptides, etc., do not form salts. The mixture of polypeptide and amino acid is boiled with copper oxide and filtered: the blue solution, if amino acids be present, is then treated with alkali and the copper oxide collected and weighed.

II. Differentiation of Enzymes.

Glycyl-l-tyrosine is readily hydrolysed by trypsin but not by pepsin. It therefore serves as an excellent compound for determining whether a given proteoclastic ferment behaves as a peptic or as a tryptic enzyme.

For this reason it was employed by Abderhalden and Rona to ascertain the nature of the enzymes contained in the pyloric and duodenal juices. Since neither of these juices acted upon glycyl-l-tyrosine they must be regarded as peptic in their nature.

Normally pancreatic juice never enters the stomach, but on a diet rich in fat bile and pancreatic enzymes may pass back into this cavity. The detection of trypsin under these conditions has been a matter of difficulty, but Abderhalden and Medigreceanu have been able to demonstrate its presence in the juice flowing from a pyloric fistula in a dog, when it was fed with oil. The juice was immediately neutralised and tested with glycyl-l-tyrosine. Tyrosine was split off. The presence of trypsin in the gastric contents in human subjects after a diet containing plentiful fat can also be demonstrated in this way if the contents are neutralised with magnesia (Abderhalden and Schittenhelm).

Abderhalden and Teruuchi used glycyl-l-tyrosine to determine the

nature of the enzymes in yeast juice (endotryptase), in papain and in the juice of nepenthes. The two former hydrolysed it and consequently they contain tryptic enzymes; the last had no action upon it, and the enzyme of nepenthes is therefore like pepsin in its action. These results confirm the observations of other investigators, and the confusion concerning the nature of these enzymes would appear to be now settled with certainty.

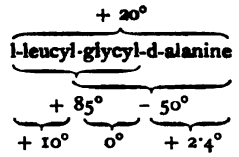
Glycyl-l-tyrosine serves only for distinguishing between a peptic and a tryptic enzyme; it is not possible to differentiate or compare the activities of the tryptic enzymes, such as pancreatic trypsin, yeast endotryptase, autolytic enzymes, etc., by its use even when the change in rotation is observed as it is hydrolysed rapidly by all these enzymes though the rates are slightly different.

For this purpose the optically active dipeptides, d-alanyl-d-alanine and d-alanyl-l-leucine were employed by Abderhalden and Koelker. By observing the change in rotation they were able to determine the rate at which these dipeptides were hydrolysed. They found that yeast endotryptase was the most active, erepsin attacked the dipeptide more slowly, and trypsin had scarcely hydrolysed them at all in forty-eight hours. The differences were not sufficiently great to allow of a differentiation.

Optically active dipeptides were also of no value for the purpose of distinguishing between the cells of normal organs, of organs in cases of cancer, and of the actual tumours; dl-leucyl-glycine, which was used as substrate and compared with glycyl-l-tyrosine and l-leucyl-diglycyl-glycine, was in all cases hydrolysed most rapidly and in all cases l-leucyl-diglycyl-glycine was most slowly hydrolysed. The same products were also formed; the dl-leucyl-glycine was split asymmetrically with the formation of d-leucyl-glycine, l-leucine and glycine.

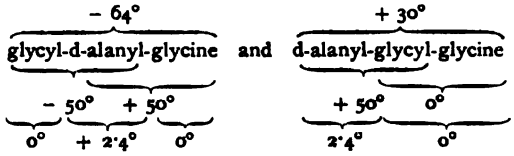
In the cases of a tripeptide, tetrapeptide, etc., the point at which the compound is first attacked may be different, and by choosing a suitable substrate the change in rotation will show at which junction hydrolysis first occurs. The first experiments in this direction were made by Abderhalden and Koelker in 1908.

The specific rotation of l-leucyl-glycyl-d-alanine is $+20°$, that of l-leucyl-glycine is $+85°$, and that of glycyl-d-alanine is $-50°$. An increase in rotation would show that d-alanine was first separated and l-leucyl-glycine formed; a decrease in rotation would point to a separation of l-leucine and the formation of glycyl-d-alanine according to the following scheme:—



It was observed, with pancreatic extract and intestinal extract together, that the rotation at first increased to an extent of about 40 per cent., and under these conditions l-leucyl-glycine must be first formed and alanine separated off. Later the rotation decreased, which was due to the hydrolysis of l-leucyl-glycine. Glycyl-d-alanine was apparently not formed at all in the process of hydrolysis. With yeast extract the rotation decreased, which showed that l-leucine was first separated.

The tripeptides glycyl-d-alanyl-glycine and d-alanyl-glycyl-glycine were also investigated and the point of first attack determined. The rotations of the various compounds are:—

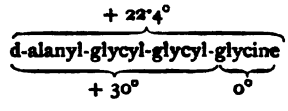


In the case of glycyl-d-alanyl-glycine, with both pancreatic extract and yeast extract, the rotation decreased at first, was reversed in direction and then again decreased in amount. Glycine and d-alanyl-glycine must therefore have been formed first and the d-alanyl-glycine must then have been subsequently hydrolysed.

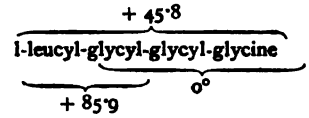
d-Alanyl-glycyl-glycine was hydrolysed by yeast extract, intestinal extract and the juices of normal tissues in such a way that the rotation gradually decreased showing that d-alanine was first separated. In the case of a cancer tumour the reverse was observed: the initial rise in rotation was followed by a fall which pointed to the separation of glycine at the commencement of the attack. Trypsin had been previously observed to effect the hydrolysis in the same way.

The action of endotryptase and of pancreatic extract upon l-leucyl-glycyl-d-alanine was next examined. With the yeast extract the rotation decreased and became negative, which showed that this tripeptide was completely split open between the leucine and the glycine. With trypsin l-leucyl-glycine was first formed and d-alanine split off.

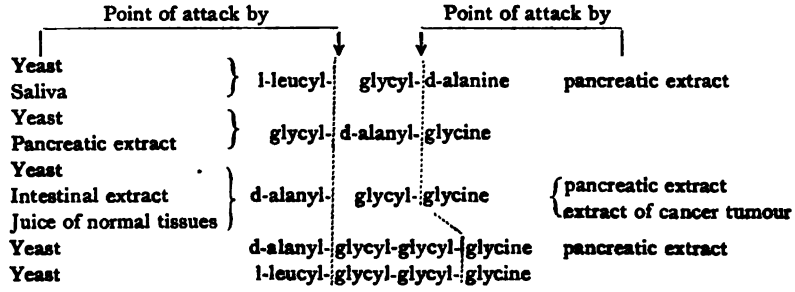
The same differences were noted with the tetrapeptide,



Yeast extract first separated d-alanine, pancreatic extract first separated glycine. Yeast extract hydrolysed



in such a way that the rotation fell, l-leucine being split off. In the hydrolysis of these polypeptides by the various enzymes we notice that yeast always separates the amino acid at the beginning of the chain. Though this occurs with most of the enzymes, yet differences in the point of attack are noticeable:—



It seems, therefore, that we possess a sharp means of differentiating between the various proteoclastic enzymes, but no certainty can as yet be placed upon the method as the variations are too few.

III. Changes in Serum under Different Conditions.

Neither the blood plasma of the horse, ox or dog hydrolyses glycyl-l-tyrosine, but this compound is hydrolysed by the blood plasma of the rabbit and guinea pig. Abderhalden and Rona extended these observations to human blood plasma in cases of disease: in some diseases no hydrolysis occurred, in other diseases there was distinct hydrolysis.

The production of anaphylactic shock by the injection of protein into the blood stream of animals has led Abderhalden and various co-workers to ascertain whether the content of the blood plasma in enzymes was influenced by injection.

Egg-white and horse serum were injected into dogs and also rabbits; subsequent examination of the plasma showed that proteoclastic enzymes were produced; they were easily detected in the dog's plasma by the hydrolysis of glycyl-l-tyrosine; the rabbit's blood plasma hydrolysed the compound more rapidly.

Injection of silk-peptone, gelatin, caseinogen and other proteins also caused the appearance of proteoclastic enzymes capable of hydro-

lysing glycyl-l-tyrosine. The enzyme is destroyed at 60° C. and is apparently given off to the plasma by the blood cells or by other organs. It is not specific for the injected protein: the plasma can hydrolyse not only glycyl-l-tyrosine, but also silk peptone and peptones prepared from gelatin, caseinogen and the other proteins by the same method as is used in preparing silk peptone (p. 71). No enzyme was produced when iodised silk peptone was injected. The serum retains its proteoclastic activity for some time after the injection, but the activity gradually decreases. No anaphylactic symptoms were produced by the injection of the silk peptone. The appearance of the enzyme in the blood stream indicates a means of protection by the animal against proteins which are not normal and not capable of assimilation when they enter the blood stream. Ferments were produced by the injection of the animal's own blood or blood from an animal of the same genus; they are produced if a large meal of protein be given and some of the incompletely digested material enters the blood stream. Serums containing diphtheria toxin and antitoxin also contain the enzyme which hydrolyses silk peptone. A mixture of the two serums did not hydrolyse the silk peptone. After injection of diphtheria toxin into dogs, the serum contained an enzyme capable of hydrolysing silk peptone. Tuberculin has the same properties. The serum of a horse suffering from pneumonia also hydrolysed silk peptone.

Proteoclastic enzymes are also found in the blood in pregnancy and seem also to be produced as a means of protection against products derived from the fœtus. The condition of pregnancy can be detected in the very earliest stages by this method.

The injection of protein into the blood does not lead to the formation of enzymes which hydrolyse carbohydrates, but these enzymes are found in the blood plasma after injection of lactose, cane sugar and other carbohydrates.

An increase in the amount of lipase in the blood plasma follows when fat is eaten in large quantities; this can be only detected by Rona's surface tension method of estimating lipolytic power.

The determination of the hydrolysis of the silk peptone and other peptones has been carried out by the polarimeter: serum and protein solution were mixed and the changes in rotation were followed. This simple method of determining enzyme action has been termed by Abderhalden the optical method. It can only be employed when a sensitive instrument is available and after considerable experience. By

its use he was enabled to ascertain if the blood plasma underwent any marked change after an animal had been bled; beyond noting a decrease in the rotation which soon disappeared no remarkable results were noticed. An attempt was made to estimate the amount of blood in the body by injecting dextrin into the blood stream and noting the change in rotation after the dextrin had completely circulated.

The detection of a proteoclastic enzyme in the blood of pregnant animals can be carried out by placing the fresh serum, prepared free from hæmoglobin, in contact with pieces of placenta which have been thoroughly washed with boiling acidulated water to remove peptones and dialysing. Peptone diffuses out and its presence can be determined by the biuret reaction, or better, by means of triketohydrindene hydrate.

IV. Study of Enzyme Action.

The optically active dipeptides form very suitable substrates for investigating the rate of action of the proteoclastic enzymes under various conditions as the changes in rotation are readily and quickly observed. They have been used by Abderhalden in conjunction with Michaelis, Gigon, Koelker, Brahm, Caemmeser and Pincussohn for this purpose. Euler has also carried out experiments of this kind. The observations conform with those of the previous observers using other methods. Further details are given in the monograph by Prof. W. M. Bayliss on the Nature of Enzyme Action.

DETERMINATION OF THE STRUCTURE OF PROTEINS.

Isolation of Polypeptides from Proteins.

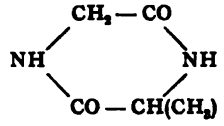
The earlier work of Fischer and Abderhalden on the digestion of proteins by trypsin, and by pepsin followed by trypsin, pointed to a combination of proline and phenylalanine in a part of the molecule, but the compound was too complex to give any real clue to the order of their combination. The first exact information as to the order of the arrangement of the amino acids was given by Fischer and Bergell in 1902, who described the formation of a dipeptide by the hydrolysis of silk-fibroin.[1]

As is well known, silk-fibroin readily dissolves in cold concentrated hydrochloric acid; if alcohol be then added, a product, called sericoin by Weyl, is precipitated, but if the silk-fibroin be allowed to stand in contact with three times its quantity of concentrated acid for about twenty-four hours, alcohol no longer produces such a precipitate, and the solution contains the hydrochloride of a peptone. Fischer and Bergell removed the hydrochloric acid from the solution of the peptone and concentrated *in vacuo;* a mass was obtained which had a bitter taste, was very soluble in water and gave strong biuret and Millon reactions, and which was very like peptone in its properties. It lost the whole of the tyrosine which it contained when it was dissolved in water and digested in ammoniacal solution with trypsin, and was converted into another peptone composed of 40·1 per cent. glycine and 28·5 per cent. alanine. Ammonia was evolved when this compound was heated with baryta water and the solution, freed from baryta, yielded crystals on evaporation; these were treated with β-naphthalene-sulphonylchloride, and a compound was obtained which was apparently β-naphthalene-sulphonyl-glycyl-alanine, though it could not be absolutely identified with the synthetical product of this composition.

The further attempts to prepare this substance again did not succeed, since the exact conditions leading to its formation could not be

[1] A small quantity of a crystalline compound was isolated in 1849 by Bopp from the products of hydrolysis of caseinogen; this was shown to be leucinimide (p. 13) by comparison with the condensation product obtained by heating leucine. Kohn's product was apparently also leucinimide.

repeated, but in 1906 Fischer and Abderhalden obtained the anhydride of this body by a new method which they had discovered for isolating such compounds when mixed with amino acids and higher polypeptides. This method depends upon the different behaviour of the esters of these compounds;[1] the esters of the simple mono-amino acids are easily volatile *in vacuo* and are therefore easily removed; the esters of the dipeptides are not volatile and are converted by the action of ammonia into their anhydrides or diketopiperazines, which crystallise readily and are therefore easily separated from the esters of the higher polypeptides. They thus obtained a methyl diketopiperazine,



which yielded glycine and d-alanine on hydrolysis and which was identical with a synthetical product prepared from glycine and d-alanine. It resulted by the hydrolysis with 70 per cent. sulphuric acid followed by trypsin, and by the hydrolysis with hydrochloric acid.

This diketopiperazine could arise both from glycyl-d-alanine and d-alanyl-glycine by the above method. It could also arise by synthesis from glycine and d-alanine under the conditions of the experiment; control experiments were carried out to determine this possibility, and they showed that this was impossible, so that there was no doubt concerning the presence of a dipeptide amongst the products of hydrolysis. Since this dipeptide was resistant to trypsin it was most probably glycyl-d-alanine and not d-alanyl-glycine, which is easily hydrolysed by this enzyme.

At the same time another diketopiperazine, glycyl-l-tyrosine anhydride, was also obtained; its nature was established a little later by identification with synthetical glycyl-l-tyrosine anhydride prepared from the ester of chloracetyl tyrosine and ammonia. In one experiment its yield amounted to 4·2 per cent. of the silk-fibroin employed.

In the same way by the hydrolysis of elastin with 70 per cent. sulphuric acid and by the action of ammonia upon the esters, a product was isolated which was composed of glycine and l-leucine and which was identical with synthetical glycyl-l-leucine anhydride.

In 1907 Fischer and Abderhalden definitely showed that the first

[1] Abderhalden and Hanslian have shown that polypeptides are not hydrolysed during the esterification process if water be carefully excluded, but proteins are hydrolysed by boiling with alcohol and hydrochloric acid.

diketopiperazine isolated from the hydrolysis products of silk-fibroin was derived from glycyl-d-alanine. Silk-fibroin was partially hydrolysed by hydrochloric acid and then precipitated by phosphotungstic acid. A portion of the filtrate from this precipitate, after removal of the excess of phosphotungstic acid, was treated in alkaline solution with β-naphthalene-sulphonylchloride and a product was obtained which was identical with β-naphthalene-sulphonyl-glycyl-d-alanine; further proof of this was given by its careful hydrolysis with dilute hydrochloric acid when the dipeptide chain was split, but the naphthalene-sulphonyl radicle not removed; β-naphthalene-sulphonyl-glycine and d-alanine were obtained according to the equation:—

$$C_{10}H_7SO_2 — NH . CH_2 . CO — NH.CH(CH_3).COOH + H_2O =$$
$$C_{10}H_7SO_2 — NH . CH_2 . COOH + NH_2 . CH(CH_3) . COOH$$

From the remainder of the filtrate glycyl-d-alanine anhydride and a small quantity of glycyl-l-tyrosine anhydride were obtained by the action of ammonia upon the esters, as well as another product which was most probably d-alanyl-l-serine anhydride.

The phosphotungstic acid precipitate contained several products of a complex nature. A substance was isolated from them which consisted of two molecules of glycine, one molecule of alanine, and one molecule of tyrosine, *i.e.*, a tetrapeptide. It had a molecular weight determined by the freezing-point method of about 350, was easily soluble in water, insoluble in alcohol, and was precipitated from its solution in flakes by saturation with ammonium sulphate or sodium chloride, as also by nitric or acetic acid. The synthetical pentapeptide, l-leucyl-triglycyl-l-tyrosine, behaves in a similar way so that great complexity, as formerly believed, is not an essential condition for precipitation by ammonium sulphate. Tyrosine was split off by the action of trypsin, and on partial hydrolysis glycyl-d-alanine anhydride and glycyl-l-tyrosine anhydride were obtained.

In the same year (1907) Fischer and Abderhalden showed by the same methods that the products of the partial hydrolysis of elastin contained:—

1. d-Alanyl-l-leucine.

2. d-Alanyl-l-leucine anhydride. This was probably formed from the above dipeptide, but since it can also be formed from the isomeric l-leucyl-d-alanine, this dipeptide may also be present amongst the products.

3. Alanyl-proline anhydride, from which d-alanine and proline were obtained on hydrolysis.

4. Glycyl-valine anhydride, which was identical in its properties, except the melting-point, with the synthetical compound.

From gliadin Fischer and Abderhalden have isolated the dipeptide l-leucyl-d-glutamic acid, which they identified with the synthetical substance; from caseinogen Abderhalden and Funk have isolated leucinimide and l-phenylalanyl-d-alanine anhydride and probably also leucyl-valine anhydride.

Osborne and Clapp obtained a crystalline substance by the acid hydrolysis of gliadin. It yielded proline and phenylalanine on further hydrolysis. Fischer and Luniak in 1909 identified it as l-prolyl-l-phenylalanine.

Levene and Beatty have isolated an anhydride, composed of glycine and proline, from the products resulting by a prolonged trypsin digestion of gelatin. This substance was optically inactive, but was again obtained by Levene in an optically active state by a shorter digestion. Its rotation, however, was still lower than the synthetical compound prepared by Fischer and Reif, but the compounds are probably identical.

Further investigations have been carried out by Abderhalden. He has isolated l-leucyl-d-alanine from the mother liquor of the elastin from which d-alanyl-l-leucine was obtained. From edestin two compounds were separated but not in a pure state; the one contained glutamic acid and tryptophane, the other contained leucine, glutamic acid and tryptophane. Neither compound was identical with the synthetical preparations which were prepared for comparison with them.

The presence of glycyl-l-tyrosine in silk-fibroin was proved by its isolation in a crystalline state in 1909.

d-Alanyl-glycine was subsequently obtained and thoroughly identified with the synthetical compound. This product can now be easily prepared as follows:—

100 grammes of silk are covered gradually with three times the quantity of 70 per cent. sulphuric acid, cooled to 0° C. Solution begins immediately and is complete in about two hours. It is then kept at 26° C. for four days, when it is diluted with 10 litres of water, the mixture being kept cold by ice. The sulphuric acid is now removed quantitatively with purified baryta and the filtrate is evaporated *in vacuo* at 40° C. The solution is tested from time to time for baryta and sulphuric acid, and if either be present it is removed. The barium sulphate is thoroughly washed with cold water and the washings concentrated in the same way. The residue obtained on complete evaporation is treated with hot methyl alcohol, in which it almost

completely dissolves. A flocculent precipitate gradually separates from the solution and is removed by filtration. The solution is again evaporated *in vacuo*, and the residue is dissolved in about 200 c.c. of water warmed to 60° C., the more insoluble portions being removed by filtration. To a test portion absolute alcohol is added until there is a distinct turbidity and to the remainder the corresponding volume required is added. On scratching the sides of the vessel and seeding the solution with a crystal of d-alanyl-glycine the compound crystallises out in about twenty-four hours. It is filtered off, washed with alcohol and ether, dissolved in water and again precipitated by absolute alcohol. The yield amounts to about 8 per cent.

The study of silk which has been undertaken by Abderhalden has shown that the different varieties contain different amounts of amino acids. They seem also to yield different anhydrides on partial hydrolysis :—

Canton silk yielded glycyl-d-alanine anhydride and glycyl-l-tyrosine anhydride. Chwang silk and Indian Tussore silk yielded d-alanine anhydride and glycyl-d-alanine anhydride. From both Canton silk and Bengal silk d-alanyl-glycine has been isolated by the above method.

In the case of tripeptides where six isomers are possible the determination of the constitution was again attempted by Abderhalden and Funk by the conjugation of the compound with naphthalene-sulphonyl-chloride. This radicle becomes attached to the amino acid at the beginning of the chain ; on hydrolysis the amino acids are separated but the naphthalene-sulphonyl radicle remains attached ; the isolation of this derivative shows which amino acid is in combination and stood originally at the beginning of the chain. For example :—

Alanyl-glycyl-tyrosine will yield naphthalene-sulphonyl-alanyl-glycyl-tyrosine which on hydrolysis will give naphthalene-sulphonyl-alanine, glycine and tyrosine. In this particular example, however, the OH group of tyrosine also reacts with the naphthalene-sulphonyl-chloride, and mono-naphthalene-sulphonyl-tyrosine will be obtained instead of tyrosine. If tyrosine stands at the commencement of the chain the product is di-naphthalene-sulphonyl-tyrosine.

The treatment of a peptone-like product from silk with this reagent and subsequent hydrolysis gave naphthalene-sulphonyl-alanine, mono-naphthalene-sulphonyl-tyrosine and glycine, from which it was concluded that alanine was at the commencement, glycine in the centre, and tyrosine at the end, *i.e.*, that the compound was alanyl-glycyl-tyrosine.

After two years of work upon this substance Abderhalden ultimately succeeded in isolating 1 gramme of this tripeptide in a pure condition. It was identified as d-alanyl-glycyl-l-tyrosine, and it is the first tripeptide which has been isolated from the products of the partial hydrolysis of proteins.

The appended list gives the polypeptides which have so far been isolated from proteins and therefore of the order in which the amino acids occur in the proteins from which they have been isolated:—

Glycyl-d-alanine anhydride	in silk-fibroin.
Glycyl-d-alanine	
Glycyl-l-tyrosine anhydride	
Glycyl-l-tyrosine	
d-Alanyl-glycine	
d-Alanyl-l-serine anhydride (?)	
d-Alanyl-glycyl-l-tyrosine	
Tetrapeptide (2 glycine + 1 alanine + 1 tyrosine)	
Glycyl-l-leucine anhydride	in elastin.
d-Alanyl-l-leucine anhydride	
d-Alanyl-l-leucine	
l-Leucyl-d-alanine	
d-Alanyl-proline anhydride	
Glycyl-valine anhydride	
Leucyl-glycine	
Gycyl-leucine (?)	
Leucinimide	in caseinogen.
l-Phenylalanyl-d-alanine anhydride	
l-Leucyl-d-valine anhydride	
l-Leucyl-d-glutamic acid	in gliadin.
l-Prolyl-l-phenylalanine	
Prolyl-glycine anhydride	in gelatin.
Dipeptide (glutamic acid + tryptophane) ?	in edestin.
Tripeptide (leucine + glutamic acid + tryptophane) ?	

The knowledge of the properties of the synthetical compounds has been of the greatest service for the isolation of polypeptides from proteins, especially the preparation of the anhydrides from the esters and the naphthalene-sulphonyl derivatives, but at present no systematic way of separating polypeptides from the mixture of complex products arising on partial hydrolysis exists. The mixture is usually divided into two fractions by precipitation with phosphotungstic acid. Each fraction, after removal of the reagent, is then fractionally crystallised or it may be treated with some other reagent, such as mercuric chloride. The identification of any product is also beset with many difficulties; its elementary composition, molecular weight and the products it yields on hydrolysis are not sufficient to establish its identity; its physical properties must be compared with those of the synthetical compound and, if necessary, derivatives may require preparation. In the case of

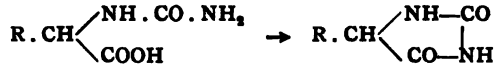
the more complex polypeptides the products of partial hydrolysis by enzymes require examination. We may, however, soon expect that the extension of the study of the higher polypeptides, more especially of the mixed polypeptides, will lead to the separation and isolation of those complexes long known as the proteoses and peptones from the products of partial hydrolysis of the proteins. Our data seem to show that they are more simple than was previously supposed, for polypeptides containing four and six units, of which tyrosine or cystine is one, can be salted out with ammonium sulphate, by which means the proteoses are now characterised.

Complete Hydrolysis after Treatment with Alkali.

Kossel and Weiss have observed that the optical rotation of the protamine, clupeine, when dissolved in semi-normal alkali, gradually fell —a rotation of $-2{\cdot}6°$ became $-1{\cdot}4°$ in sixty-four hours and $-0{\cdot}5°$ in seven days at the ordinary temperature, a rotation of $-2{\cdot}75°$ became $-0{\cdot}51°$ in fifty hours at a temperature of 38° C., and the rotation of a gelatin solution under the same conditions fell from $-5{\cdot}84°$ to $-0{\cdot}57°$ in fourteen days at the ordinary temperature. This change in rotation was not due to the hydrolysis of the compound and the subsequent racemisation of the amino acids; there was only slight hydrolysis and arginine, the chief constituent of clupeine, does not undergo such a marked change in rotation when treated with alkali. The only conclusion which could be drawn was that racemisation of the arginine occurs very readily whilst it is in combination in the protein. Subsequent hydrolysis of the protein yielded dl-arginine and dl-ornithine. The same result was obtained with gelatin.

An explanation of this phenomenon has been given by Dakin:—

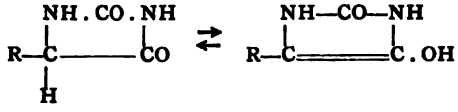
Uramido acids are converted by mineral acids into hydantoins:—



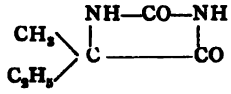
The optical rotation of the uramido acids in alkali is constant; the rotation of the optically active hydantoin diminishes and becomes zero; and the hydantoin obtained on acidifying is optically inactive.

The racemisation process is connected with the group

which can undergo tautomeric change:—

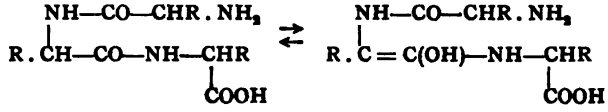


This supposition was proved by an examination of the optically active methyl-ethyl hydantoin,



prepared from isovaline by the action of potassium cyanate. It suffered no racemisation on treatment with alkali.

Amino acids in combination possess a structure similar to the hydantoins and can undergo this keto-enol tautomerism as follows:—



Only one of the asymmetric carbon atoms can undergo this change: the carbon atom which remains unaffected is at the end of a chain of amino acids.

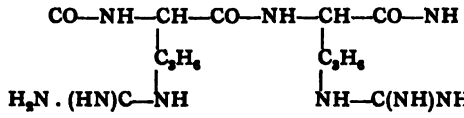
Hydrolysis of a protein after treatment with alkali should yield a mixture of optically inactive and optically active amino acids. By ascertaining which of the amino acids are active the end members of a chain will be ascertained. Dakin's preliminary results[1] with gelatin show that lysine, glutamic acid and a part of the alanine and proline are obtained in an optically active form; the leucine, aspartic acid, phenylalanine, histidine and arginine were entirely inactive.

Lysine and glutamic acid would thus appear to be at the end of a chain of amino acids.

[1] Dr. Dakin has kindly pointed out this phenomenon to me and informed me of these results of his experiments, which have since been published.

Complete Hydrolysis after Treatment with Nitric Acid, Nitrous Acid, etc.

Kossel and Kennaway showed that clupeine could be nitrated and converted into nitroclupeine. Nitroarginine was obtained on hydrolysis. The nitration of arginine occurred in the guanidine part of the molecule. Consequently the guanidine grouping will not be used in the linking of amino acids, but will remain free :—



By treating proteins with nitrous acid and then hydrolysing the product with acid Skraup was unable to separate lysine from the mixture of amino acids. Its ϵ-amino grouping was apparently destroyed by the nitrous acid, so that most probably this grouping is not used in the linkage of amino acids. Further evidence that this amino group is not used in linkage is given by the experiments of Kossel and Cameron upon the action of nitrous acid on sturine by van Slyke's method. An amount of nitrogen was evolved which corresponded very closely to the amount of lysine in the molecule and it corresponded to only one of its two amino groups.

Kossel and Gawrilow have also shown that the ϵ-amino group of lysine is free and not used in the linkage of the amino acids by the reaction of proteins with formaldehyde. Those proteins containing very little or no lysine only react very slightly or not at all. In general, although the proportion is not exact the more lysine a protein contains the more it reacts with formaldehyde.

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